Immune responses of the insect Manduca sexta towards the bacterium Photorhabdus luminescens

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Immune responses of the insect *Manduca sexta* towards the bacterium *Photorhabdus luminescens*

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Dedicated to Mum, Dad and Sarah

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Abstract

The Gram-negative bacterium *Photorhabdus luminescens* is a pathogen of insects. It is able to secrete a variety of toxins and effectors against its host in order to escape its immune defences. The model insect *Manduca sexta* is able to mount a variety of humoral and cellular responses against pathogen attack. Ultimately these prove ineffective against *P. luminescens*. The pre-treatment of *M. sexta* with *Escherichia coli* provides protection against the pathogenesis of *P. luminescens.* Here, I use RNA interference and Fluorescence-assisted cell sorting techniques to investigate interactions between pathogen and host to further elucidate the roles of various host factors in mounting the immune response. I also investigate the nutrient requirements of the bacteria for pathogenesis. I show data that peptidoglycan recognition protein (PGRP) is essential for the up-regulation of antimicrobial peptides, an important immune defence. I also show that *P. luminescens* has a requirement for two types of iron during pathogenesis of *M. sexta*. And lastly I show that *P. luminescens* is able to avoid phagocytosis, another important immune defence.

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Abbreviations

°C – degrees Celsius

% - Percent

AMP – Anti-Microbial Peptide/Protein

BC – Before Christ

βGRP – β-1,3-Glucan Recognition Protein

BSA – Bovine Serum Albumin

CCTV – Closed-Circuit Television

cDNA – Complementry DNA

CFU – Colony-Forming Units

CT – Baseline-subtracted Threshold

DAP – Meso-diaminopimellic acid

DHC – Differential Haemocyte Count

DHR - Dihydrorhodamine

DMPC – Dimethyl pyrocarbonate

dsCON – dsRNA for a control gene: A plant catalase

dsF – dsRNA specific for Ferritin

dsHEM – dsRNA specific for Hemolin

dsPGRP – dsRNA specific for PGRP

dsRNA – Double-Stranded RNA

dsTF – dsRNA specific for Transferrin

EDTA – Ethylenediaminetetraacetic acid

FACS – Fluorescent-Activated Cell Sorting

FHV – Flock House Virus

FITC – Fluorescecein Isothiocyanate

FSC-H – Forward Scatter

G – Maximum Relative Centrifugal Force or G-Force

g – Grams

GIM – Grace's Insect Medium

GFP – Green Fluorescent Protein

GNBP – Gram-Negative Bacteria-Binding Protein

GSB - Glutathione-S-Bimane

h – Hour(s)

IMD – Immune Deficient

IML - Immulectin

IJ - Infective Juveniles

kDa – KiloDalton

JAK – Janus Kinase

LacZ – Beta-D-galactosidase

LB – Luria-Bertani

LD_{50} – Lethal Dose, 50% or median lethal dose

LPS – Lipopolysaccharide

MAMP – Microbial-Associated Molecular Pattern

MCB - Monochlorobimane

µg – Micro-grams

µL – Micro-litres

µM – Micro-molar

mg - Milligrams

mL – Millilitres

mM – Milli-molar

mRNA – Messenger RNA

M – Molar

NCBI – National Centre for Biotechnology Information

NGF – Nerve Growth Factor

NLR – Nod-Like Receptor

nm – Nanometres

nt - nucleotides

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PGN - Peptidoglycan

PGRP – Peptidoglycan Recognition Protein

PI – Propidium Iodide

PNA – Peanut Agglutinin

PO - Phenoloxidase

PPO – Prophenoloxidase

PRR – Pattern Recognition Receptor

PSP – Plasmatocyte Spreading Peptide

PTC - Phenylthiocarbamide

RIP – Receptor Interacting Protein

RNAi – RNA interference

ROS – Reactive Oxygen Species

rpm – Revolutions Per Minute

RT – Reverse Transcriptase

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SSC-A – Side Scatter

ssRNA – Single-Stranded RNA

ST – (*E*)-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl)benzene

TAE – Tris-Acetate EDTA

TEM – Transmission Electron Microscope

THC – Total Haemocyte Count

TLR – Toll-Like Receptor

Tm – Melting Temperature

TNFR – Tumor Necrosis Factor Receptor

TPBS – Tween Phosphate Buffered Saline

Upd-3 – Unpaired-3

WGA - Wheatgerm Agglutinin

Ybt – Yersinabactin

Chapter 1 – Introduction

Immunity, first described by Thucydides during the plague of Athens in the year 430 BC (Retief and Cilliers, 1998), is the ability of an organism to recognise and defend itself against non-self, particularly disease. Disease is caused when pathogenic micro-organisms attack host cells seeking resources so that they may grow, or when normally benign micro-organisms are introduced to a different environment and cause havoc. As such, many organisms from bacteria to humans have developed some sort of an immune system to defend themselves from pathogens and other potential harm-inducing objects, which works around the principle of discriminating non-self from self and neutralising it. The immune system in most plants and animals is made up of two or three lines of defence: surface, innate and adaptive. The components of each of these lines of defence are outlined in Figure 1.1.

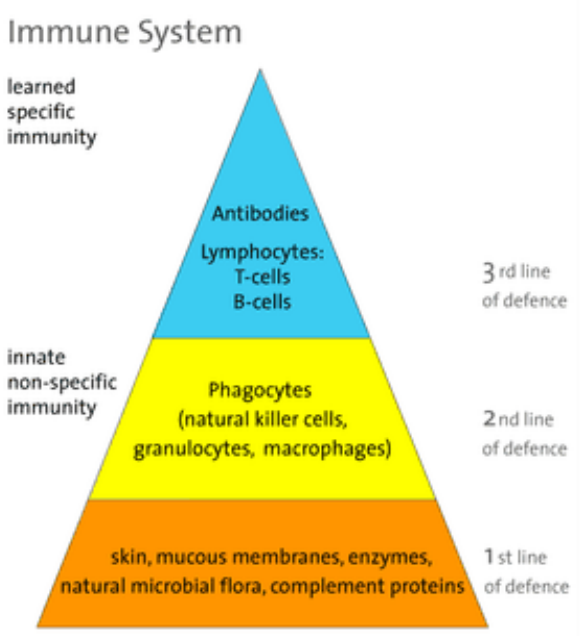

Figure 1.1 – A diagram showing the components of each line of defence in the immune system (http://www.infections.bayer.com/en/bacteria/immunesystem/index.html).

There are many components involved in the immune system that work synergistically to protect against infection (Parham, 2005). It starts with a physical barrier such as the skin or the waxy cuticle of leaves which prevents the internal tissues from being exposed to pathogens. The chemical nature of these surfaces

together with secretions from both the host and pre-existing micro-flora is often hostile towards invaders. The pre-existing micro-flora will also out-compete the pathogen for available resources. Routes into the body are similarly protected by the resident micro-flora and by mucus membranes which line the respiratory and digestive tracts. Mucus is continually produced by these membranes which traps invading pathogens. Stomach acid will quickly kill most micro-organisms that escape the mucus. Again, the resident micro-flora within the gut will out-compete most pathogens that survive long enough to get there. However, despite all these barriers, some pathogens are able to penetrate and start to cause an infection. This is where remaining lines of defence that make up the humoral and cellular responses come into play against the pathogen.

Innate vs. adaptive immunity

Anything that the immune system identifies as non-self is known as an immunogen. Therefore any molecule (provided that it is above a certain molecular size) that is present on the microbial cell surface is potentially an immunogen and can trigger the humoral and cellular responses of the immune system. Such immunogenic molecules include polysaccharides, lipids, proteins and nucleic acids. The cellular response, as it name implies is largely to do with cells responding to the presence of an immunogen. This is typically phagocytosis of the invading micro-organism. The humoral response is chemical in nature and involves proteins and other agents that inhibit pathogens or aid the cellular response in phagocytosis. Typical products of the humoral response include anti-microbial peptides and antibodies. Both of these responses contain specific and non-specific elements. The specific element is known as adaptive or learned immunity, while the non-specific element is known as innate immunity. Innate immunity is a fast maximal response to the presence of an immunogen. It recognises the immunogen as non-self and initiates the production of inhibitory substances such as interferon, complement or anti-microbial peptides and also attracts phagocytes. The innate immune response varies to only a limited extent according to the nature of the immunogen, i.e. it is relatively non-specific. In insects, for example, immune responses do differ between responses to Gram negative bacteria and those to Gram positive bacteria and fungi (Lemaitre and Hoffmann, 2007). There is, however, considerable cross-talk between these two immune signalling pathways.

Adaptive or learned immunity is a slower response. When an immunogen is first encountered by the adaptive system (this mostly occurs by parts of the innate system presenting the immunogen to it), the challenged animal selects from a pre-existing range of cells to find one with a receptor that 'fits' the immunogen best. That cell then synthesises secreted antibodies with the same recognition domain as the selected antigen receptor. That is to say the selected cell produces antibodies that match the immunogen. This selected cell then proliferates and mass produces the antibody until the infection is overcome; subsequently, most of these cells will die away, but a few will remain to form a 'memory' of that particular infection. If that particular pathogen is ever re-encountered, then these 'memory' cells will be activated very quickly to mass-produce once again, and counter the pathogen more effectively. Hence this is why the adaptive or learned immunity is so called, because it 'adapts' to the pathogen and 'learns' to recognise it, (see Kindt et al., 2007). However, adaptive immunity is specific to one type of pathogen and the animal will have to learn again even if the new pathogen is similar to another pathogen that was encountered previously. Adaptive immunity has, however, up until very recently considered to be found only in vertebrate animals, and so most organisms including insects depend on the innate immune system to recognise and defend against disease. More recently, however, it has been reported that at least in mosquitoes, adaptive immunity may occur through selection of particular patterns of post-transcriptional processing of the mRNA encoding a particular immune-related protein, Dscam (Garver et al., 2008). It is not yet clear if a similar system of alternative splicing of this protein is involved in immunity in other insects, although homologous genes are found in the genomes of *Drosophila* and other insects. It should be mentioned, however, that there have been claims of apparently adaptive responses in a few insects. The extent to which these responses represented a genuinely adaptive reponse is uncertain.

For example in *Periplaneta americana* immunization with killed *Pseudomonas aeruginosa* induced significant ($P < 0.05$) two-week long protection against this bacterium (Faulhaber and Karp, 1992) The response's specificity was tested by immunizing with other killed bacteria (*Serratia*, *Enterobacter*, *Streptococcus* or *Micrococcus*), and then challenging them with live *P. aeruginosa*. Significant protection was induced by any of the bacteria within the first 3 days after injection.

However, the immunity to the *P aeruginosa* challenge elicited by the other bacteria declined more quickly than that due to *P. aeruginosa* itself. The authors claimed that this represented a two stage response with specificity in the second stage, but other interpretations are possible, most notably that *P. aeruginosa* is simply much better at "priming" the insect's immune system than the other bacteria, perhaps because it persists longer within the insect.

A study of "primed" immune responses in *Drosophila melanogaster* (Pham et al., 2007) provides a rather more convincing case of apparent specificity (the authors describe this specificity as "crude"). Here, injecting killed *Streptococcus pneumoniae* elicited prolonged protection against this bacterium, but not against other lethal bacteria (*Salmonella typhimurium*, *Listeria monocytogenes*, and *Mycobacterium marinum*). In turn, injecting killed cells of these bacteria did not protect against *S. pneumoniae*. A similar specific protective response was elicited by infection with the entomopathogeneic fungus *Beauveria bassiana*, and this response did not cross react with that to *S. pneumoniae*. These responses, which used the Toll pathway of immune signalling, appeared not to depend on elevated levels of circulating AMPs, but on cellular responses that could be blocked by inhibiting phagocytosis with polystyrene beads. In this study, therefore, it was convincingly showed that a degree of immune specificity exists in the responses of flies to two different pathogens; however, the extent of the specificity remains only partially explored. It is not known whether insects can distinguish among strains of the same species of bacteria, for example, as can the mammalian immune system.

Another immune phenomenon that is currently under-explored is the transfer of immunity between parent and offspring in insects. There are well documented examples of this in bumble bees (Sadd et al., 2005) and in the mealworm, *Tenebrio molitor* (Moret, 2006). In the last case, what was measured was an antimicrobial response in the offspring following an immune challenge (injection of LPS) to the larval stage of parental generation. This could be a case of simple, but very long lasting priming of the parental immune system, together with the non-speficic transfer of haemolymph proteins into the egg. Since transgenerational immunity is of some interest to evolutionary ecologists, however, it seems worthwhile to probe the mechanism further.

Insects as model organisms

Insects are proving to be useful in the study of disease and immunity amongst other studies including social behaviour and genetics. There are a number of reasons why insects make good models for these studies. Firstly, insects are easy to produce and the maintenance of colonies easier than with mammals, secondly, the ethical issues of using insects in research are reduced compared to mammals. Thirdly, at least some insects are genetically tractable, and have fully sequenced genomes. Finally, the similarities between some systems including the innate immune system are close enough to enable direct comparisons to be made.

In mammals, studying the innate immune response to infection is problematic because as explained above the non-specific responses work synergistically with the adaptive responses to provide a very effective response to pathogens. Therefore it is not possible to study the innate immune response in mammals or other vertebrates without allowing for the effect of the adaptive responses on the same pathogen. Insects, however, as mentioned above, have at best a very limited adaptive immune response and are therefore highly suited for studying innate immune responses to infection (Muller et al., 2008).

In addition some insect species cause huge amounts of damage to crops worldwide while others are vectors for diseases that affect plants, animals and humans. The developing world, in particular, is vulnerable to these diseases so studying how insects respond to pest control techniques is key to alleviating these problems.

Many insect species have been used for studies, some have commercial importance such as *Bombyx mori* (Silkworm) or *Apis mellifera* (Honey Bee), others are important vectors of disease such as *Anopheles gambiae* (Mosquito) or *Glossina morsitans* (Tsetse Fly) but most work has been done, particularly in immunity, in the fruit fly *Drosophila melanogaster*. This is because this species is easy to manipulate genetically, reproduces quickly and as it has a published genome, easy to search for homologous or putative immune genes. In fact, *D. melanogaster* was where the Toll gene was first discovered by Christiane Nüsslein-Volhard in 1985 (Hansson and Edfeldt, 2005), although the gene was originally discovered on account of its role in embryogenesis, rather than its role in immunity. It was only ten years later that Jules Hoffmann's group discovered its role in immunity (Lemaitre et al., 1996). Following

this, mammalian homologues were discovered in 1997 by Ruslan Medzhitov and Charles Janeway (Medzhitov et al., 1997), which activate the adaptive immune response. Shortly after, it became apparent that the mammalian Toll-like receptor 4 (Tlr4*)* was a receptor for Lipopolysaccharide (LPS) a common constituent of bacterial cell walls (Poltorak et al., 1998) and therefore formed part of the pattern recognition receptors group of proteins, a vital part of the immune system.

Manduca sexta (Tobacco Hornworm) is a lepidopteran insect that is used extensively in research, particularly for physiological and biochemical studies (Kanost et al., 2004). Its large size (the fifth instar typically reaches 10-12 g), makes this insect easy to manipulate physically, enabling easy injection of substances. This also allows specific doses of bacteria to be injected, allowing the calculation of LD_{50} (Mahajan-Miklos et al., 2000). Another advantage to the large size of *M. sexta* is the ability to collect between 1-2 mL of haemolymph (containing approximately 10^6 haemocytes) from each insect meaning that fewer insects are required for experiments. This large size also means that it is feasible to do biochemical experiments on immune related body chemicals, such as haemolymph proteins, which can be purified from biological samples.

Insect immunity

It is increasingly obvious that insect immunity is much more complex than was originally thought, and in this general review of the insect immune system, I will restrict my account to aspects that are relevant to the experimental work that is described in the main body of the thesis.

Pattern recognition proteins

When confronted with a microbial challenge *M. sexta* and other insects mount an immune response comprising both humoral and cellular components, which work synergistically to try and counter the threat (Gillespie et al., 1997) The first step of this response is being able to detect the presence of a microbe.

Similar to a CCTV surveillance system, certain proteins are responsible for detecting microbial-associated molecular patterns (MAMPs) that are as the name suggests, common to microbes but absent from their host (Hoffmann et al., 1999). Such proteins form part of a group known as pattern recognition receptors (PRRs). PRRs bind to a particular MAMP and it is these complexes that initiate the immune

response. Several PRRs have been identified in *M. sexta* and these include β-1,3-glucan recognition proteins (βGRPs), hemolin, immulectins and peptidoglycan recognition proteins (PGRPs) (Yu et al., 2002)

Two βGRPs, βGRP-1 and βGRP-2, have been found in *M. sexta* (Ma and Kanost, 2000, Jiang et al., 2004). These bind to β-1,3-glucan, which is a cell wall component of fungi, and also lipoteichoic acid, a cell wall component of gram-positive bacteria. This results in agglutination of bacteria (both gram-negative and gram-positive) and yeast, and results in stimulating the activation of prophenoloxidase (PPO), an important insect antimicrobial response (Ma and Kanost, 2000, Jiang et al., 2004). It is not clear how both βGRPs are able to agglutinate gram-negative bacteria as neither bind to peptidoglycan or LPS (Jiang et al., 2004). βGRP-1 mRNA is synthesised in the fat body tissue of *M. sexta* at a constitutive level that does not change if the insect is injected with yeast or bacteria. βGRP-1 protein is found in the haemolymph of *M. sexta* (Ma and Kanost, 2000). Likewise βGRP-2 is found in the haemolymph of *M. sexta* but is also present in the cuticle (Jiang et al., 2004). βGRP-2 mRNA is also synthesised in the fat body but according to Jiang et al. (2004) rather than being constitutively expressed it is induced by the injection of yeast and bacteria, although it is also developmentally upregulated when the insect reaches the prepupal or wandering stage (Jiang et al., 2004).

The two βGRP proteins share a 57% identity in their amino acid sequence (Jiang et al., 2004) and contain a carboxyl-terminal glucanase-like domain and a less-conserved amino-terminal domain which is responsible for the strong binding of these proteins to β-1,3-glucan, akin to the βGRP found in *B. mori* (Ochiai and Ashida, 2000, Kanost et al., 2004). The carboxyl-terminal glucanase-like domain is so-called because of its similarity to the β-1,3-glucanases found in bacteria, but actually lacks enzymatic activity due to substitutions of key amino acids within the catalytic site. Other arthropod species have similar proteins with a carboxyl-terminal glucanase-like domain that are catalytically inactive, suggesting conservation of this particular protein. These include *B. mori* (Ochiai and Ashida, 1988, Ochiai and Ashida, 2000), *Blaberus craniifer* (Death's Head Cockroach) (Soderhall et al., 1988), *Pacifastacus lenusculus* (Freshwater Crayfish) (Lee et al., 2000) and *Plodia interpunctella* (Indian Meal Moth) (Fabrick et al., 2003). In *Blaberus discoidalis* (West Indian Leaf Cockroach), a lectin has been shown to recognise β-1,3-Glucan

(Chen et al., 1995). All of these βGRPs have been shown to induce the PPO cascade. Some insects have proteins with a glucanase-like domain which are also able to recognise Gram-negative bacteria and are thus termed Gram-negative bacteria-binding proteins (GNBP). GNBPs bind to surface moieties of bacteria as well as β-1,3-Glucan (Jiang, 2008) and also have been shown to enhance antimicrobial gene expression in *D. melanogaster* (Kim et al., 2000).

Hemolin is a 47 kDa protein that binds to LPS and lipoteichoic acid, components of Gram-negative and Gram-positive bacteria respectively (Yu and Kanost, 2002). It appears to be exclusive to lepidopteran insects, having been found in *Hyalophora cecropia* (Rasmuson and Boman, 1979), *Hyphantria cunea* (Fall Webworm) (Shin et al., 1998), *Lymantria dispar* (Gypsy Moth) (Lee et al., 2002) and *M. sexta* (Ladendorff and Kanost, 1990), while no ortholog has been found so far in the genomes of *D. melanogaster* or *A. gambiae*. The protein itself is composed of four I-set immunoglobulin domains to create a horseshoe-shaped structure (Su et al., 1998, Yu et al., 2002). As well as binding to bacteria, hemolin also has the ability to bind to haemocytes (Zhao and Kanost, 1996) suggesting that hemolin has a dual role in bacterial defence; to act as a PRR and modulate haemocytic responses. This ability to bind both bacteria and haemocytes also lends to the suggestion that the protein could act as an opsonin, increasing the efficiency of phagocytosis (Zhao and Kanost, 1996) and in fact the knockdown of hemolin by RNA interference (RNAi) has been shown to reduce both phagocytosis and nodule formation in response to an injection of *Escherichia coli* (Eleftherianos et al., 2007b). A recent study (Labropoulou et al., 2008) has shown that hymenopteran parasitoid wasp larvae may interfere with hemolin function when they parasitize their lepidopteran hosts, and the authors concluded that their results fully confirmed the findings of Eleftherianos et al., (2007b).

Hemolin in *H. cecropia* has been shown to bind to LPS (Daffre and Faye, 1997), and to haemocytes in a calcium-dependent manner (Bettencourt et al., 1999). The horseshoe-like structure and the interaction between the immunoglobin domains of hemolin in *H. cecropia* have suggested a model of homophilic bonding of hemolin proteins that are present on haemocyte or microbial surfaces (Su et al., 1998). Hemolin could have two binding sites for LPS, one that interacts with carbohydrates found in the O-antigen and outer core regions, and another that interacts with

phosphate groups found in the lipid-A component (Yu and Kanost, 2002). It is suggested that the phosphate interaction site of hemolin is also responsible for the binding of the protein to lipoteichoic acid (Yu et al., 2002), as this MAMP is largely made up of poly(glycerophosphate) chains linked to membrane phospholipids (Fischer et al., 1990).

The strong induction of hemolin mRNA and protein synthesis in both fat body and haemocytes by bacterial challenge has been shown in both *M. sexta* (Ladendorff and Kanost, 1990, Eleftherianos et al., 2006a, Eleftherianos et al., 2007b) and *H. cecropia* (Rasmuson and Boman, 1979). Injection of double stranded RNA (dsRNA) for hemolin (dsHEM) or a control dsRNA reagent also caused upregulation of hemolin in *Antheraea pernyi* (Chinese Oak Silk Moth) probably due to it having a role in the anti-viral response (Hirai et al., 2004), however, in *M. sexta*, no such upregulation was found. Injecting dsHEM into *M. sexta* resulted in increased susceptibility to the insect pathogens *Photorhabdus asymbiotica* and *Photorhabdus luminescens* TT01 (Eleftherianos et al., 2006a, Eleftherianos et al., 2006b). Hemolin synthesis has also been found in embryos (Bettencourt et al., 2000) and also during metamorphosis of naive insects (Yu et al., 2002), perhaps suggesting a role in development or perhaps just defending the insects when they happen to be particularly vulnerable. Further evidence for a role in development is provided with the observation that RNAi knockdown of hemolin in *H. cecropia* pupae is lethal to the next generation of embryos (Bettencourt et al., 2002).

Four immulectins (-1 to -4) have been found within *M. sexta* (Yu et al., 2002). They belong to a superfamily of C-type lectins, which are calcium-dependednt carbohydrate-binding proteins and have functions in pathogen recognition, cellular interactions and innate immunity in mammals (Weis et al., 1998, Vasta et al., 1999, Yu et al., 2002). A group of 19 C-type lectin genes have been found in *D. melanogaster* but their function has yet to be determined (Theopold et al., 1999). Lectins in other insect species have been found and shown to participate in various immune functions including phagocytosis (Jomori and Natori, 1992) and the activation of PPO (Chen et al., 1995). The four immulectins (IML) of *M. sexta* contain 2 tandem C-type lectin carbohydrate recognition domains (Yu et al., 1999, Yu et al., 2002). Similar proteins have been found in 2 other lepidoteran insects, *B. mori* (Koizumi et al., 1999) and *H. cunea* (Shin et al., 2000, Shin et al., 1998). This

is in contrast to most other animals where the C-type lectins contain only 1 carbohydrate domain (Yu et al., 2002).

The four IMLs of *M. sexta* are differentially regulated and also differ in their ligand specificity (Yu et al., 2002). IML-1 is undetectable in haemolymph plasma, but increases in response to microbial infection (Yu et al., 2002). It has been shown to bind both Gram-negative and Gram-positive bacteria and yeast and will cause these micro-organisms to aggregate. (Yu et al., 1999) Very little is known about IML-3 and -4. The concentration of IML-3 increases in response to microbial infection but IML-4 remains at the same level (i.e. it remains constitutively expressed) (Yu et al., 2002). The microbial ligand specificity of both IML-3 and IML-4 remains unknown, but have been shown to bind to *N*-acetylgalactosamine and glucose (Yu et al., 2002).

The most well known and the best studied *M. sexta* IML is IML-2. It is constitutively expressed but is up-regulated upon infection with Gram-negative bacteria or injection with LPS (Yu and Kanost, 2004). Once bound to LPS, IML-2 stimulates activation of PPO by binding to serine proteases present in haemolymph plasma (Kanost et al., 2004). IML-2 has been shown to be important in the clearance of a Gram-negative bacterium, *Serratia marcescens*. The injection of an IML-2 antibody inhibited the clearance of *S. marcescens* from the haemolymph of the insect and this resulted in decreased survival (Yu et al., 2002). Knock down of IML-2 by RNAi results in increased susceptibility to *P. asymbiotica* and *P. luminescens* (Eleftherianos et al., 2006a, Eleftherianos et al., 2006b). Unlike hemolin and PGRP, IML-2 was not found to be expressed by haemocytes following a microbial challenge (Eleftherianos et al., 2007b).

PGRP is a PRR that binds to peptidoglycan (PGN). PGN is a polymer made up of chains of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid linked together by β-1,4 linkages (Lemaitre and Hoffmann, 2007). These chains are cross-linked to form a thin sheet by peptides consisting of four amino acids attached to *N*-acetylmuramic acid. The composition of the cross-linking peptides differs in bacterial species, whereas the polysaccharide backbone rarely changes. In Gram-negative bacteria, the third amino acid in the peptide is always meso-diaminopimelic acid (DAP), whereas in Gram-positive bacteria, the third amino acid is generally lysine, although in *Bacillus* species it is DAP. This third amino acid determines the

complexity of the cross-link with DAP; the cross link is usually direct to the D-alanine on the other peptide involved in the cross-link. Those PGNs that have lysine or another amino acid, however, have an interpeptide bridge that varies in its make-up depending on the species involved. PGN is unique to bacteria and is responsible for maintaining the shape of the cell and the osmotic pressure within it. Gram-negative bacteria have a thin layer of PGN beneath the LPS outer membrane, while Gram-positive bacteria contain several layers of PGN within their cell walls. It is these factors that make it an important trigger and target of immune responses. The first PGRP was discovered in *Bombyx mori* (Yoshida et al., 1996) and since then, many homologues have been found in other animal species, including many mammals. In addition, there have other immune recognition proteins found that also recognise peptidoglycan including CD14, Toll-like receptor (TLR) 2 and Nod 1 + 2, members of the Nod-like receptor (NLR) family (Royet and Dziarski, 2007).

Several animal species have been found to have more than one from of PGRP, *Homo sapiens* (Humans) and *Mus musculus* (Mouse) have four and *Euprymna scolopes* (Hawaiian Bobtail Squid) also has four. Insect PGRPs are also often alternatively spliced to give more variety as shown by the malaria mosquito *Anopheles gambiae*, which has seven PGRP genes spliced into nine proteins. This is also seen in the fruit fly *Drosophila melanogaster*, which has no fewer than 13 genes spliced into 19 proteins (Royet and Dziarski, 2007).

The functional significance of the multiple forms of PGRP is not well understood. But studies on *Drosophila* have gone some way towards revealing their functions. Each PGRP is differentially expressed in response to different stimuli, presumably each isoform performing one or specific tasks against the invading bacteria (Lemaitre and Hoffmann, 2007). The extent to which the tasks are distinct or overlapping is not known. Nevertheless, some generalizations can be made.

PGRPs generally fall into one of two forms: Long or Short. Table 1.1 lists all the forms that are currently known in *D. melanogaster*.

Drosophila melanogaster PGRPs	
Long forms	PGRP-LA-C PGRP-LA-D PGRP-LA-E PGRP-LA-F PGRP-LB-A PGRP-LB-B PGRP-LB-C PGRP-LC-A PGRP-LC-B PGRP-LC-C PGRP-LD-A PGRP-LD-B PGRP-LD-C PGRP-LE PGRP-LF
Short forms	PGRP-SA PGRP –SB1 PGRP-SB2 PGRP-SC1a PGRP-SC1b PGRP-SC2 PGRP-SD

Table 1.1 – A list of the short and long forms of peptidoglycan recognition proteins currently known in *Drosophila melanogaster*. (Adapted from (Royet and Dziarski, 2007)

The –LA isoforms are expressed in haemocytes, and their function has yet to be determined. The –LB isoforms are expressed in fat body and gut and they function as amidases. Amidases are able to hydrolyze peptidoglycan into smaller pieces. As will become evident below, both catalytically active and inactive forms of PGRPs are also able to function in a signal transducing role. The –LC isoforms are expressed on the surfaces of haemocytes and fat body and are responsible for initiating the *IMD* pathway. –LC-A also has a role in phagocytosis. The –LD isoforms are expressed in haemocytes and their function has yet to be determined. The –LE protein is expressed in the gut, haemocytes, and trachea and has a role in activating both the *IMD* pathway and PPO. Finally, expression and function of the –LF protein have yet

to be determined (Royet and Dziarski, 2007). The –SA protein is expressed in fat body, haemolymph and epidermis and is involved in initiating the Toll pathway, it acts as a carboxypeptidase and is involved in phagocytosis. The –SB isoforms function as amidases, -SB1 is expressed in fat body while expression of –SB2 has yet to be determined. The –SC isoforms are expressed in the gut, with –SC2 also being expressed in fat body. They all function as amidases with –SC1a also involved in activation of the Toll pathway and phagocytosis. The –SD protein is expressed in fat body and is involved in Toll activation (Garver et al., 2006).

As described above, some PGRPs are catalytically active and function as amidases. Actually all the PGRPs share a domain with similarity to bacteriophage T7 lysozyme, a zinc-dependent *N*-acetylmuramoyl-L-alanine amidase, which serves the recognition site, but those regarded as catalytically inactive lack the zinc-binding residues that are needed for amidase activity (Zaidman-Remy et al., 2006). PGRPs with amidase activity can decrease or abolish the biological activity of peptidoglycan by cleaving part of or all of the peptide from the sugar backbone. Nevertheless, as we will now discuss, catalytically active PGRPs may either upregulate or downregulate immune responses.

One of the best understood examples of the class of catalytically active PGRPs is PGRP-SC1a. This PGRP form is essential for initiating the Toll pathway and phagocytosis when *D. melanogaster* is infected with the Gram-positive bacterium *Staphylococcus aureus*. A mutant known as *picky* is unable to produce PGRP-SC1a, and so when *picky* mutants are infected with *S. aureus* these insects will die quicker than the wild-type flies (Garver et al., 2006). By contrast, when the *picky* mutant is injected with Gram-negative *Escherichia coli* or *Bacillus subtilis,* the *IMD* pathway of innate immune defence is evoked and phagocytosis occurs as normal. This reveals that PGRP-SC1a is specific for the Lysine-type peptidoglycan that is typical of most Gram-positive bacteria. Analysis reveals that the Toll pathway is not activated and that no phagocytosis occurs in *picky* mutants when challenged with *S. aureus*. However, this phenotype can be rescued when PGRP-SC1a protein is introduced back into the *picky* mutant (Garver et al., 2006). Moreover, further experiments reveal that the catalytic activity of PGRP-SC1a is differentially important for the two immune responses that are elicited by this PGRP. When a catalytically inactive PGRP-SC1a is used to rescue the *picky* mutant, infection with *S. aureus* now causes

activation of the Toll pathway, but not phagocytosis, showing that cleavage of the peptide is necessary for Toll activation but not phagocytosis. However, introducing free peptidoglycan rescues the catalytically inactive PGRP-SC1a phenotype by inducing phagocytosis of *S. aureus*, indicating that it is the release of free peptidoglycan following PGRP cleavage of Lys-type peptidoglycan by PGRP-SC1a that causes phagocytes' activation (Garver et al., 2006).

In contrast, another well understood form, PGRP-LB is reported to act as a downregulator of the immune response (Lemaitre and Hoffmann, 2007). An issue for *D. melanogaster* is that overactivation of the *IMD* pathway can lead to developmental defects or apoptosis so it becomes necessary to limit or curtail the immune response to prevent this. Also due to the lifestyle of *D. melanogaster*, which involves close contact with bacteria and other micro-organisms in their food, sometimes a false warning will be set off, which can be detrimental due to the reasons explained above. PGRP-LB is mainly expressed in the gut, but also can be found in the haemolymph, and recognises DAP-type peptidoglycan (Royet and Dziarski, 2007). It cleaves this form of peptidoglycan making the peptidoglycan molecules inactive and unable to stimulate the immune system. It has been suggested that the reason for this is the association of *D. melanogaster* with commensal bacteria and other relatively harmless bacteria from its diet of rotting fruit. These bacteria will contain peptidoglycan within their cell walls, and so *D. melanogaster* will need to deactivate this within the gut to prevent this activating the immune response (Girardin and Philpott, 2006). Moreover, because peptidoglycan fragments will also inevitably cross the gut epithelium and enter the haemolymph, a further level of defence is required. It is supposed that the amidase activity of PGRP-LB present in the haemolymph will also act to prevent initiation of inappropriate immune responses by cleaving the fragments to render them inactive (Lemaitre and Hoffmann, 2007).

But catalytically inactive PGRPs are also important in immune responses (Lemaitre and Hoffmann, 2007). One of these, PGRP-LC is expressed on the surface of fat body cells and haemocytes and is required for the activation of *IMD* pathway (Royet and Dziarski, 2007). Mutations in PGRP-LC result in the insect being unable to produce the antimicrobial peptides that are regulated by the *IMD* pathway, these insects are highly vulnerable to Gram-negative bacterial infection. PGRP-LC

recognises DAP-type peptidoglycan typical of Gram-negative bacteria and of *Bacillus* species, in both polymeric and monomeric forms. Two alternatively spliced isoforms, PGRP-LCa and PGRP-LCx are responsible for this recognition. A third alternatively spliced form, PGRP-LCy has also been found but as yet, its ligand and role are unknown (Dziarski, 2004). PGRP-LCx has been shown to be required for the detection of polymeric peptidoglycan and both PGRP-LCx and PGRP-LCa are needed to recognise monomeric peptidoglycan. The proteins form heterodimeric structures which is then able to start the signalling cascade that ultimately results in the activation of the *IMD* pathway.

PGRP-LE is expressed in gut, haemocytes and in trachea, and can be found both extra- and intracellularly (Dziarski, 2004). The extracellular form is smaller than the intracellular form and only consists of the PGRP domain. Its function is seemingly to assist peptidoglycan recognition by PGRP-LC although the exact mechanism is yet to be determined (Royet and Dziarski, 2007). The intracellular form primarily deals with monomeric DAP-type peptidoglycan that enters the cytoplasm by mechanisms unknown. Studies that either overexpress or knockout PGRP-LE reveals that this isoform is involved in the activation of the PPO cascade.

In summary, through the recognition by various forms of PGRP of either DAP-type or Lys-type peptidoglycan, *D. melanogaster* is able to respond to infection by bacteria through the activation of both the Toll and *IMD* pathways and also prevent false activation by commensal or ingested bacteria. It should be noted that activation of the Toll and *IMD* pathway by LPS remains controversial. Studies by the group of Bruno Lemaitre have shown that commercial preparations of LPS are often contaminated with PGN and that it is this PGN, not LPS, that is actually responsible for activation of Toll and *IMD* pathways., Use of PGN-free preparations of LPS does not activate these immune signalling pathways (Lemaitre and Hoffmann, 2007).

The phenoloxidase system

One of the most studied components of the insect immune system is phenoloxidase (PO), the enzyme that converts phenolic precursors into reactive precursors of melanin (Cerenius et al., 2008, Marmaras et al., 1996, Nappi and Christensen, 2005). The enzyme exists in haemolymph plasma as an inactive precursor, prophenoloxidase (PPO); when MAMPS are detected, the PPO is converted to the

active form by proteolytic cleavage; in *Manduca* the components of the PPO activating complex have been studied in great detail by Michael Kanost and his colleagues (reviewed by Kanost et al., 2004). At least some aspects of PPO activation can be reconstituted in vitro using purified components (Gupta et al., 2005, Wang and Jiang, 2007). Despite this detailed understanding, what happens before this final step is less well understood in any insect. The activation of the PPO cleaving enzyme is the consequence of a poorly defined proteolytic cascade. In *Manduca*, one haemolymph component that recognises MAMPs and activates the cascade has been identified as the protease HP14, but this does not exclude the possibility of others (Lu and Jiang, 2007). At least three different proteases that can cleave PPO to activate it are known. Two of these are PPO-activating proteinases 2 and 3, and these are both known to be activated in turn by the same enzyme, haemolymph serine proteinase 21 (Gorman et al., 2007, Wang and Jiang, 2007). It is not known whether the PPO-activating proteinases are differentially activated under different kinds of immune challenge. It is likely that the activation of this signalling cascade in *Manduca* is restrained by one or more endogenous serine protease inhibitors (Wang and Jiang, 2004). In *Drosophila*, the serpin Necrotic is known to be involved as a negative regulator of PPO activation (Pelte et al., 2006).

Regardless of the exact method of PPO activation, it is increasingly clear that PO really does play an important role in the defence of the insect against bacteria. Numerous comparative studies of ecological aspects of immunity have measured PO activity in insect haemolymph, revealing that PO activity is closely correlated with the ability to resist infection (Siva-Jothy et al., 2005). Experimental demonstrations of the importance of PO include a study by Eleftherianos et al. (2007a) in which it was shown that the insect pathogenic bacterium *Photorhabdus luminescens* secretes a small molecule, (*E)*-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl)benzene, (ST) that inhibits activated PO. Genetic manipulation of the bacterium to prevent ST secretion decreased virulence. Moreover, RNAi knock down of infection-induced PPO expression showed that the effect of ST on virulence required the presence of PPO.

Antimicrobial proteins and peptides

Insects were first shown to produce antimicrobial peptides and proteins (AMPs) by the work of Boman and his colleagues in the 1960s, who experimentally infected diapausing pupae of the silkmoth *Hyalophora cecropia* and showed that the resulting antibiotic activity in the insect's haemolymph was due to the secretion of cecropin, a peptide that disrupts bacterial cell membranes (Boman and Steiner, 1981) as well as establishing the production of attacins and lysozyme. Since then a very large number of AMPs have been described from various insects (Hancock et al., 2006). Their sequences and structures are listed, along with AMPs from other organisms, in a publicly available database: http://www.bbcm.units.it/~tossi/antimic.html

Most insect AMPs have been found to be strongly upregulated by infection or exposure to MAMPs. The increased circulating level of AMPs is the basis of the protection to subsequent infection that is conferred by previous exposure to harmless bacteria (Eleftherianos et al., 2006b) or fungi (Bergin et al., 2006). The extent to which different AMPs are upregulated depends on the nature of the microbial pattern that is experienced, and this implies that the existence of different immune signalling pathways. Thus in *Drosophila*, the predominant AMP expressed on challenge by Gram negative bacteria are Diptericin, Drosocin, and Attacin, regulated by the Immune Deficient (IMD) signaling pathway, whereas the response to challenge by Gram Positive bacteria or fungi results in the expression of the AMPs Drosomycin and Metchnikowin, regulated by the Toll signaling pathway (reviewed by Lemaitre and Hoffmann, 2007 – see below for further discussion of immune signaling). It will be shown in Chapter 3 that exposure of *Manduca sexta* caterpillars to infection by Gram negative bacteria results in the upregulation of several AMPs. Further it will be shown in Chapter 5 that the levels of AMPs that are present in such "primed" insects is sufficient to kill most *Photorhabdus* cells when these were injected 18h after pre-exposure to injected *E. coli* cells.

Iron sequestration

Microbial pathogens generally need to grow and multiply within the host in order to cause disease, and require many of the same nutrients as host cells. Therefore competition between the cells of host and parasite frequently occurs for those nutrients that are found at only low concentrations in animal body fluids. Iron is an

excellent example of such a limiting resource; because iron is essential for most microorganisms during an infection, both animals and plants have evolved immune defenses that limit the availability of iron to pathogens (Andrews et al., 2003).

Iron has only low solubility in the presence of carbonate and phosphate ions, and is present (usually as FeII ions) in aqueous solution in animal blood and tissue fluids at only very low concentrations. Since Iron is an almost universal requirement by living cells to enable oxidative metabolism, evolution has equipped all living cells with mechanisms to acquire iron, usually involving the use of iron-chelating molecules to bind iron and transport it across cell membranes. Thus microorganisms use a range of small molecule siderophores, while animals use the iron transport protein transferrin to do the same thing. Acquiring iron from the environment is only part of the story, however; because iron in solution in the presence of oxygen catalyses the production of reactive oxygen radicals through the Fenton reaction (Ong et al., 2006) the activity of stored iron within cells must be reduced. This is achieved in animals through a different iron binding protein, ferritin.

These same mechanisms used for iron uptake and storage can also be used to sequester iron within the body so that its activity of iron is reduced to levels so low that pathogenic microorganisms cannot take it up. The basic mechanism is to increase the concentration of transferrin in blood plasma. Transferrin has an extremely high affinity for iron, and when it is present in excess, the activity of free iron in solution falls to such low levels (~10^{-18}M – (Wandersman and Delepelaire, 2004)) that the body fluids are in effect iron-free. Once bound by transferrin, the chelated iron is then taken up by cells and stored by binding to ferritin. This iron-withholding immune strategy in the face of microbial infection is extremely common among animals (Ong et al., 2006).

Insects are not unusual in this iron-withholding strategy, and a range of insects have been shown to upregulate transferrin production when subjected to an immune challenge (Law, 2002). Thus, when *Drosophila* is exposed to septic injury, mRNA levels of two transferrin genes are increased suggesting that iron sequestration in the control of microbial development (Yoshiga et al., 1999). Similar upregulation of transferrin expression has also been shown in mosquitoes (Yoshiga et al., 1997).

Cellular responses

The main effectors of cellular immune responses in insects are the blood cells or haemocytes. These cells vary considerably between insects, but in lepidopterans are mainly described as granular cells, plasmatocytes, spherule cells and oenocytoids (Lavine and Strand, 2002, Price and Ratcliffe, 1974, Ribeiro and Brehelin, 2006, Strand, 2008). Many studies have been made of haemocyte counts, either as the total haemocyte count (THC) or differentially according to type (DHC). It is well attested that both THC and DHC change rapidly during immune responses to infection or challenge with MAMPs (Au et al., 2004, Dean et al., 2004b, Lackie, 1988). The significance of such changes is not well understood, however. Haemocytes are usually characterised by their appearance in the light microscope, especially after they have been allowed to adhere a microscope slide. This approach is laborious and slow, however. Ribeiro and Brehelin advocate the use of transmission electron microscopy (TEM) to identify haemocyte types, but this is even slower and very expensive and can hardly be considered a practicable approach for cell counting.

The use of monoclonal antibodies to identify haemocyte types would certainly help in this type of study (Willott et al., 1994) but the extent to which such antibodies are able to recognise the same type of cell among a range of different insects is uncertain. Even to suggest that this would be desirable raises the question of what would be meant by the "same type of cell" when considering two different species of insect. An important point, seldom addressed however, is that a significant proportion of haemocytes may at any one time not be in circulation, but sessile within the tissues (Ratcliffe et al., 1985). This is a potential complication, since small difference in the protocol used to harvest haemocytes may affect the results obtained.

Haemocytes participate in a number of immune responses directed against invading microorganisms (Lavine and Strand, 2002; Strand 2008). These are phagocytosis (the active cellular engulfment of microorganisms); nodule formation (in which host cells adhere to large groups of invading microorganisms and to each other, so as to form a cellular coating around it); and encapsulation (essentially the same as nodule formation, but generally applied where the invading parasite is larger, for instance in the case of insect parasitoids). The identities of the cells that take part in these activities seems to be quite variable among insects, which leads to concern that the

process of classification into cell types on morphological grounds may not be as useful as it seems. In *Manduca sexta*, the main phagocytic cell type appears to be a specialised type of plasmatocyte, the hyperphagocytic cell, and this cell type may also initiate nodule formation (Dean et al., 2004b). Other lepidopterans differ however, and in *Galleria mellonella*, granular cells are the main agents in both processes (Ratcliffe and Gagen, 1976). Strand (2008) commented that "the main capsule forming haemocytes in Lepidoptera are plasmatocytes but studies in several species indicate that granular cells are also present".

Typically, nodule formation and encapsulation are accompanied by PPO activation and the production of a black layer of melanin on the surface of the surrounded parasite or pathogen. It is usually supposed by this, that melanisation is actively detrimental to the microorganism, but a direct demonstration of this is lacking. It is also possible that the principal function of melanin deposition on the surfaces of microbes and/or parasites might actually be to promote the adherence of haemocytes, as in opsonisation. As noted above, prevention of melanisation has a deleterious effect on the insect's ability to resist infection (e.g. Eleftherianos et al., 2006b) but it should be noted that inhibition of melanisation might also have deleterious effects on the process of nodule formation and encapsulation. Although a number of authors have noted that oenocytoids are the main quantitative source of circulating PPO, and these cells release the enzyme when they are damaged (reviewed by Strand, 2008), it is not certain that oencytoid-derived PPO is responsible for the melanising activity that occurs during nodule formation and encapsulation.

The adherence of haemocytes to surfaces is regulated by a peptide that is released following immune challenge, the plasmatocyte spreading peptide (PSP). In the armyworm, *Pseudoplusia includes*, PSP promotes plasmatocyte adhesion and spreading, but inhibits these activities in granular cells (Strand and Clark, 1999). The peptide appears to have additional roles, however, which include some adverse consequences to the insect (blockage of growth, even death) when too much peptide is present (Strand et al., 2000). A homologue of PSP is present in *Manduca sexta* and has similar activities (Wang et al., 1999). Experiments in our laboratory have shown that RNAi knock down of the PSP gene result in impaired cellular responses to infection by *P. luminescens* (I. Eleftherianos et al., unpublished – personal communication).

Nodule formation and associated PPO activation are also promoted by lipid signalling involving eicosanoids (Dean et al., 2002, Miller, 2005). At present the tissue or cellular type that is the source of these eicosanoids remains uncertain.

In Chapter 5 I address a technological advance that may facilitate studies on insect cellular responses. Methods are developed to measure the extent of phagocytosis in vivo of GFP-expressing bacteria. Identification of the types of cell undertaking the phagocytosis should ultimately be possible. This may prove helpful in rapid characterization of immune responses and evaluation of the effect of experimental manipulation (e.g. RNAi knockdown experiments) on such cellular responses.

Cellular immune signalling

Regulation

Genetic studies in *Drosophila* have led to the discovery of two cellular immune signaling pathways that are obviously very important in immune gene expression, and another one that is apparently of lesser importance (Ferrandon et al., 2007, Lemaitre and Hoffmann, 2007, Tanji and Ip, 2005). One of these, primarily involved in responses to fungi and Gram-positive bacteria, was named after the gene *Toll*, already known for its role in early embryonic development. A second pathway, primarily involved in signaling for responses to Gram-negative bacteria, involves the gene *Immune deficient* (*IMD*). This gene was not previously known for any non-immune role and was discovered through a screen for the immune related expression of AMPs. Microarray studies of transcript levels in *IMD*/*Toll* double mutant flies have shown that these pathways regulate almost 80% of immune related genes. These two pathways are illustrated schematically in Figure 1.2

A third pathway, the JAK/STAT pathway appears to control the expression of a smaller subset of effector genes. There are almost certainly interactions between all three pathways, which may additionally be developmentally regulated (Lemaitre and Hoffmann, 2007).

Both the Toll and IMD pathways employ similar, although distinct steps in their later stages, involving movement from the cytoplasm into the nucleus of NF-κB-like transcription factors (Lemaitre and Hoffmann, 2007). Three NF-κB/Rel-like

proteins, Dorsal, Dif and Relish, are encoded in the *Drosophila* genome; all three bind to κB sites in gel shift assays and are able to activate transcription of AMP mRNAs in cultured cells. Promoter mapping experiments with *Drosophila cecropin* and *diptericin* have shown, as expected, that the genes encoding these AMPs are regulated by nuclear transcription factors. Several upstream regions of DNA are required for immune inducibility, notably including κB binding sites (Engstrom et al., 1993, Senger et al., 2004).

It was quickly realized after the discovery of *Toll*'s involvement in immunity that this role has been evolutionarily conserved from the earliest multicellular eukaryotes. Even plants have Toll-like immune related genes (Jordan et al., 2002). Moreover, Toll-like receptors (TLRs) in mammalian cells control immune responses to a variety of different elicitors. The IMD pathway also appears to be conserved, being homologous to the mammalian Tumor necrosis Factor Receptor (TNFR) pathway (Lemaitre and Hoffmann, 2007).

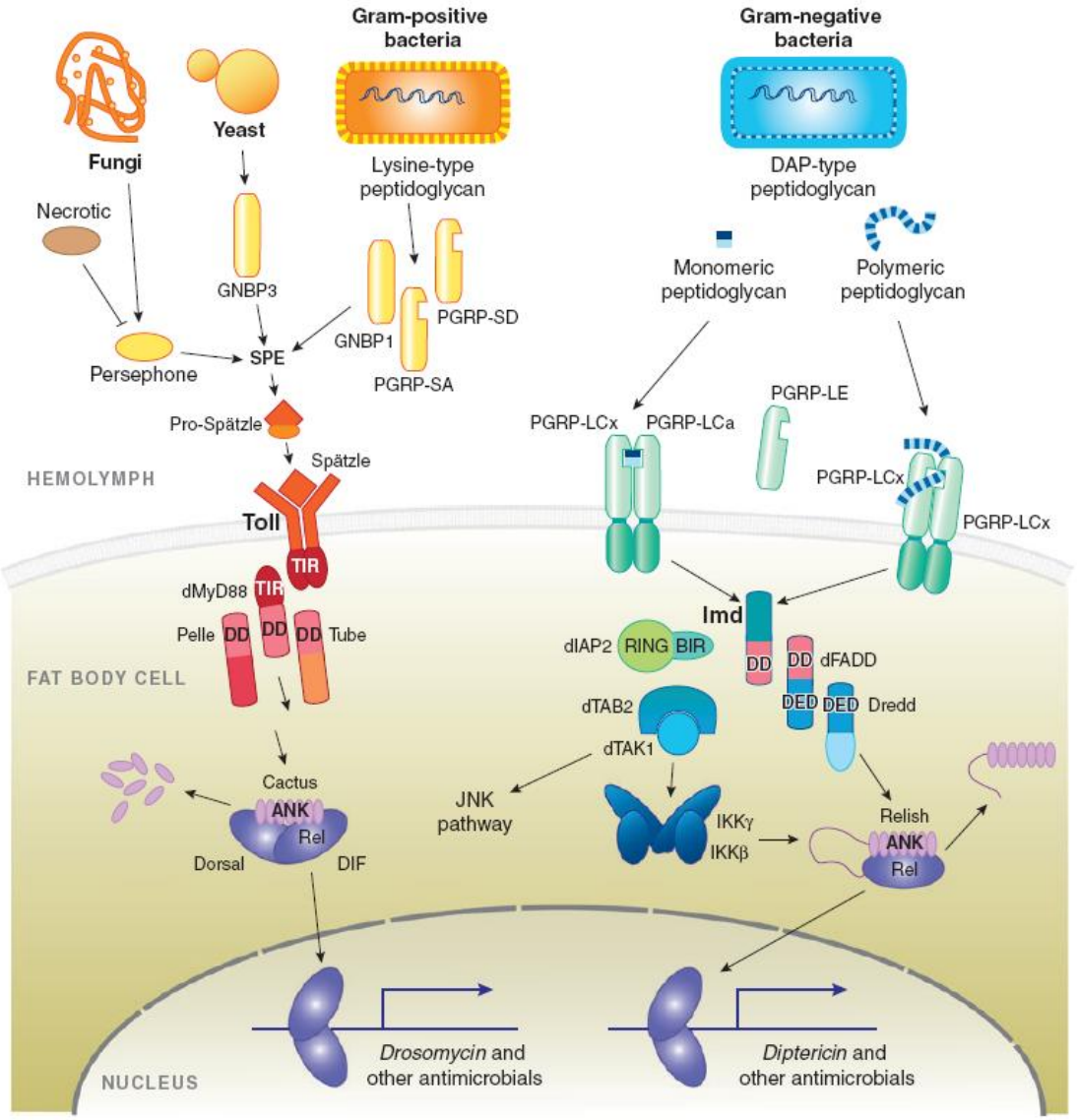

Figure 1.2 - Cellular immune signaling in *Drosophila melanogaster*, showing the principal components and interactions of the Toll and IMD pathways. See text for description and further details. Reproduced from Lemaitre and Hoffmann, 2007.

The Toll pathway

Toll is a plasma membrane protein and acts as a receptor. Although eight other Toll proteins have been identified in the *Drosophila* genome (Lemaitre and Hoffmann, 2007), only Toll itself is clearly involved in regulating immunity. The ligand that activates Toll is the nerve growth factor (NGF)-like cytokine Spätzle, which circulates in haemolymph (Lemaitre and Hoffmann, 2007). Toll forms a receptor complex with the Death domain containing proteins Tube, Pelle and MyD88. Toll signaling is initiated by proteolytic cleavage of Spätzle, which is catalyzed by the

serine protease Spätzle processing enzyme SPE (Kambris et al., 2006). Since several upstream signaling pathways apparently converge at this point, SPE evidently acts as an integrating factor. Interaction between dimeric, cleaved Spätzle and Toll causes receptor dimerization (Hu et al., 2004), thereby activating the protein kinase Pelle, which then phosphorylates Cactus (the *Drosophila* homolog of IκB), a protein that forms part of the heterotrimeric complex with Dorsal and Dif. Once phosphorylated, Cactus is degraded by proteasome action, releasing Dorsal and Dif to enter the nucleus and to transactivate expression of Drosomycin and other AMPs (Lemaitre and Hoffmann, 2007).

An important difference between *Drosophila* Toll pathway and its mammalian Toll-like counterpart is that whereas TLRs act as membrane receptors for MAMPs, *Drosophila* Toll does not bind directly to these elicitors. Instead, the state of Spätzle is regulated by an upstream mechanism involving PRRs (short form PGRPs, and the confusingly named Gram-negative binding proteins [GNBPs] which actually bind fungal β-glucans as well as surface moieties of bacteria (Jiang, 2008), serine proteinases (e.g. Persephone) and serpin (see above) (reviewed by Lemaitre and Hoffmann, 2007).

The IMD pathway

This pathway was originally discovered (Lemaitre et al., 1995) because mutations in a gene named *immune deficiency* (*IMD*) adversely affect expression of Diptericin and several other AMP genes but only marginally affect induced expression of Drosomycin. *IMD* deficient flies are vulnerable to Gram-negative bacteria but are relatively resistant to fungi and Gram-positive bacteria. Unlike Toll, IMD is not a membrane protein. Instead, *IMD* encodes a Death Domain–containing protein similar to the Receptor Interacting Protein (RIP) of the mammalian tumor necrosis factor receptor (TNFR) pathway (Georgel et al., 2001). Thus IMD is more like MyD88 than Toll. One membrane receptor that initiates the IMD pathway has been identified as a long form PGRP (PGRP-LC). It is activated by binding to monomeric or polymeric DAP-type peptidoglycan (Gottar et al., 2002). The molecular detail of the IMD pathway is less well understood than the Toll pathway, but a number of molecular components are known and mutants in any of these generate an *IMD*-like phenotype. As would be expected, overexpression of IMD causes constitutive

expression of AMPs. Like the Toll pathway, the crucial step is the nuclear localization of an NF-κB family protein, Relish, probably as a result of proteolysis affected by the caspase, DreDD (Leulier et al., 2000). Once translocated, Relish acts as a transcription factor to promote the formation of AMP mRNAs (Silverman et al., 2000).

The JAK/STAT pathway

Studies on the malaria mosquito, *Anopheles gambiae*, originally revealed that in addition to the Toll and IMD pathways, the JAK/STAT pathway also plays a role in immune responses (Barillas-Mury et al., 1999). This signaling route, originally identified through its role in embryonic development, is much simpler in operation than the other two pathways. Three main cellular components, the receptor Domeless, the Janus Kinase (JAK) Hopscotch, and the STAT transcription factor, are involved. Occupation of the cell surface receptor Domeless leads to activation of the Janus Kinase (JAK) Hopscotch, and the phosphorylation of the STAT transcription factor causes its nuclear translocation and consequent upregulation of a subset of *Drosophila* immune-responsive gene. These include genes encoding the complement-like protein *Tep2* and the *Turandot* stress genes (Ekengren and Hultmark, 2001); although the control of these genes is complex and interactions exist with other signaling pathways (Brun et al., 2006).

The significance of the immune role of the JAK/STAT pathway is less clear than the Toll and IMD pathways. There is significant cross-talk with other signaling pathways including the IMD pathway (Delaney et al., 2006). JAK/STAT-deficient flies are not more susceptible to bacterial and fungal infections but are less resistant to viral infection (Dostert et al., 2005). The nature of the ligand that activates this pathway during infection is not certain. Unpaired-3 (Upd-3) is a cytokine-like protein that is released from haemocytes during immune challenge; it activates the JAK/STAT pathway by binding to the Domeless receptor in fat body cells (Agaisse et al., 2003). It was suggested that Upd-3 and Domeless may constitute a danger system (Matzinger, 1994) that responds to tissue damage.

Photorhabdus luminescens

P. luminescens is a virulent pathogen of insects that is an obligatory symbiont of soil dwelling heterorhabditid nematodes (Forst et al., 1997). The bacteria live in a quiescent state within the nematode gut, and do not attack the worm. The nematode seeks out an insect host and invades it by mechanically disrupting the insect's body surface cuticle, thus gaining entry to the haemocoel. The bacteria are now able to kill the host (only a small number of bacteria, in the order of 100 – Forst et al., 1997 - are required to kill the host). Even before the host is dead they begin to multiply, following which the nematodes are able to use host resources too and also proliferate. When the insect's body tissues have been consumed, an unknown environmental signal causes the bacteria to be swallowed and repackaged by the nematodes, now in the form of "infective juveniles" (IJs). These leave the host cadaver, and seek out a new host, thus renewing the life cycle (illustrated in Fig. 1.3). (ffrench-Constant et al., 2003).

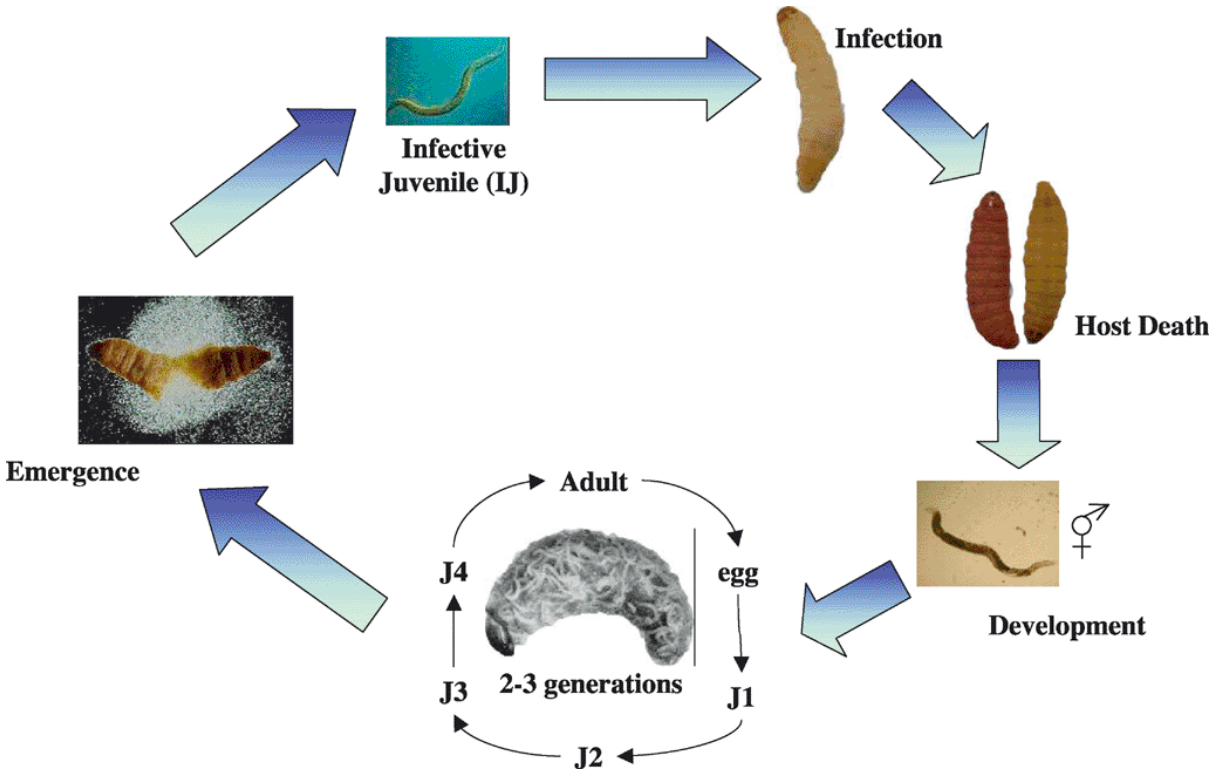

Figure 1.3: Lifecycle of *Photorhabdus luminescens*. Reproduced from ffrench-Constant et al., (2003).

Early in the infection process, once inside the host insect, the symbiotic bacteria are released from the nematode gut. It is at this stage that the bacteria are recognised by the host insect's immune system, and antimicrobial defences are deployed against them. It is known that *Photorhabdus* employs a number of anti-immune defences, including the ability to prevent host phagocytosis and to secrete toxins that kill host cells including haemocytes (ffrench-Constant et al., 2007a). Nevertheless, despite these defences, the number of bacteria recoverable from the infected insect, inside the first 24 h of infection, falls to a very low level (Au et al., 2004). Work in our laboratory (Eleftherianos et al., 2006a) has shown that insect host immune defences play a significant role in restraining the extent and speed of bacterial pathogenesis; RNAi disruption of insect immune genes results in the insect becoming even more susceptible to *Photorhabdus* than normal. Further, preinfection by a harmless Gram-negative bacterium is able to "prime" the *Manduca* immune system so that *Photorhabdus* virulence against these primed insects is reduced compared to naïve controls (Eleftherianos et al., 2006b). Some of the work contained in this thesis contributed to these papers, and the results are reported and discussed in greater detail in Chapter 3.

There are several species in the genus *Photorhabdus* (Fischer-Le Saux et al., 1999). All are entomopathogenic, but only *P. asymbiotica* is able to cause human disease. All have nematode partners. The relationship between the bacterial symbiont and the nematode host is a very close one, and particular isolates of bacteria can only co-exist with their own nematode. This effectively prevents extensive genetic exchange, and different strains of *Photorhabdus* show considerable differences in the phenotypic and genotypic characters. In the present work, I have used exclusively the TT01 strain of *P. luminescens*, which is genetically stable, easy to work with, and for which the complete genomic DNA sequence data is available (Duchaud et al., 2003).

At this early stage of infection, *Photorhabdus* takes up a specialised position on the inner (haemocoel) surface of the insect's gut (Silva et al., 2002). Subsequently, the bacteria begin to proliferate, and the host dies, presumably due to the secretion by the bacteria of sufficient toxins and hydrolytic enzymes to disrupt host physiology beyond normal limits. The success or otherwise of the bacteria in resisting host defences and subsequently proliferating within it depends on the balance of attack

and defence between the two organisms. This balance may depend in part on their relative abilities to sequester nutrients from the haemolymph. In Chapter 4, I describe some work in which the ability of host and parasite to sequester iron was separately experimentally manipulated. This depended on the availability of genomic information for the TT01 strain of *Photorhabdus*. The outcome of these experiments revealed that the ability of the bacterium to grow within the host is critically dependent on certain genes that are required for iron uptake. Interestingly, only one of these same genes is required for bacterial success in a different insect, *Galleria mellonella* (Watson et al., 2005). This reveals that the critical balance of particular host and parasite defences differs according to the host species.

Photorhabdus is able to detect when it is present within the insect and a large number of genes are specifically upregulated when the bacterium is in contact with insect extract (Munch et al., 2008). These genes include a number that are thought to encode toxins. Analysis of the genome sequences of two different *Photorhabdus* species (Duchaud et al., 2003, Waterfield et al., 2008) has shown that these bacteria are extremely well provided with putative toxins. These toxins almost certainly contribute to the ability of this bacterium to overcome host immune defences. Among them are the Tc toxins that exert cytotoxic effects against a wide variety of host cells, but which are probably mainly active against gut epitheleial cells. These toxins are extremely complex and it appears that the high molecular weight active protein toxins are assembled from the products of several genes (Ffrench-Constant and Waterfield, 2006). Other toxins include Mcf and Mcf2, toxins that provoke programmed cell death in host cells (Dowling et al., 2004), and LopT, a toxin that inhibits phagocytosis and which is secreted direct into target cells through a Type III Secretion System (Brugirard-Ricaud et al., 2004). The *mcf* gene is alone capable of conferring on normally non-pathogenic *E. coli* the ability to persist within and kill *Manduca* caterpillars (Daborn et al., 2002). Other less-well studied toxins from *Photorhabdus* include Txp40 (Brown et al., 2006) and Pir (Blackburn et al., 2006). In Chapter 5, I will report experiments which show that *Photorhabdus* toxins prevent its own phagocytosis when the bacterium is injected into *Manduca*, and that *Photorhabdus* is also able to prevent the phagocytosis of injected *E. coli.*

There continues to be much interest in the interaction between *Photorhabdus* and its insect hosts as a tractable invertebrate model of bacterial pathogenicity (Ffrench-

Constant et al., 2007b, Joyce et al., 2006, Silva et al., 2002). Furthermore, some of the insecticidal toxins encoded by *Photorhabdus* genes may eventually find a use in agriculture, perhaps through the genetic engineering of crop plants (Ffrench-Constant, 2007).

This thesis further explores selected aspects of the interaction between *Photorhabdus luminescens* and *Manduca sexta* by investigating the following:

- The response of the PRR peptidoglycan recognition protein (PGRP) towards infection by *P. luminescens* and it's role in the defence of *M. sexta* against the pathogen
- The role of transferrin and ferritin, if any, in the immune defence of *M. sexta* against *P. luminescens*, and what strategies are employed by the microbe to sequester iron from it's host
- The development of a protocol for fluorescent-activated cell sorting (FACS) to study haemocyte population changes in response to infection from *P. luminescens*

Chapter 2 - Materials and methods

Manduca sexta (Tobacco hornworm) culture

M. sexta larvae were raised in an insectarium which maintained a 25^{o}C temperature, 50% humidity environment with a 17 hours light: 7 hours dark photoperiod on an artificial wheatgerm based diet (Reynolds et al., 1985). First instar caterpillars were individually placed on a piece of diet and left for 12-14 days until they reached the fifth instar. The insects were then transferred to a larger block of diet, and left for 4-5 days until they reached the wandering stage. Wanderers were then placed into wooden blocks for 10 days to pupate. After pupation, the pupae were taken out of the wooden blocks and left for 10-15 days until ready to hatch. The pupae were then put into a separate cage and left to emerge. The adult moths were fed with a 10% sucrose solution and water daily. The adults would lay their eggs onto a nappy liner, which was changed daily also. These eggs were placed back in the insectarium to renew the cycle.

Bacterial cultures

Stock bacterial cultures

Bacterial stock cultures were maintained under 20% glycerol at -80°C using standard procedures.

Escherichia coli culture

E. coli strain DH5α was used for all experiments. Five milli-litres (mL) of Luria-Bertani (LB) (recipe in Appendix 1) medium broth was inoculated with 5 µL of a 20% glycerol stock culture of *E. coli* and then incubated overnight at 37^{o}C in a shaking incubator at ~220 rpm.

The *E. coli* culture was prepared for injection by removing a 1 mL aliquot from the overnight culture into a 1.5 mL microcentrifuge tube and centrifuging it at 17,000 G for 5 minutes. The supernatant was then removed and the pellet resuspended in 1 mL of Phosphate buffered saline (PBS) (recipe in Appendix 1). The insects were injected with 50 micro-litres (µL) of this suspension.

To calculate the number of colony-forming units (CFU) injected into each caterpillar, the resuspended bacterial culture was serially diluted five times. Thirty micro-litres of the 10^{-4} and 10^{-5} dilutions were plated out on to LB medium containing 1.5% agar plates (recipe in Appendix 1) and incubated at 37°C overnight. The CFU on each plate was counted and then the number of CFU present in the resuspended bacterial culture was worked out by the following formula:

$$\text{Number of colonies on dilution plate} \div$$

$$(Dilution\ factor \times Amount\ of\ diluted\ sample\ used\ to\ spread\ plate\ (in\ mL))$$

Finally, the resulting number from the equation above was divided by 0.05 to give the number of CFU injected into the insects, this was typically 1.1 x 10^6 bacteria.

***Photorhabdus luminescens* culture**

P. luminescens strain TT01 was used for all experiments. Cultures were prepared by streaking LB medium containing 1.5% agar plates with 20% glycerol stock culture of TT01 and incubating the plates at 28°C in the dark for 2 days.

Overnight cultures were then prepared by inoculating 5 mL of LB medium broth with a single colony from the plate and then incubated at 28°C in a shaking incubator at ~220 rpm.

The TT01 culture was prepared for injection by removing a 1 mL aliquot from the overnight culture into a 1.5 mL microcentrifuge tube and centrifuging it at 17,000 G for 5 minutes. The supernatant was then removed and the pellet resuspended in 1 mL of PBS. The cultures were then diluted to 2 x 10^4 (for experiments in Chapter 4) or 2 x 10^3 (for experiments in Chapters 3 and 5) cells per mL in the following way: The absorbance reading at 600 nanometres (nm) was taken for the resuspended culture, and the number of cells was estimated from this value; this culture was diluted to 2 x 10^8 cells per mL accordingly and subsequently serially diluted.

Similarly to the injection of *E. coli*, 50 µL of the serially diluted culture was injected into the caterpillars each time. This meant that insects in Chapter 3 and 5 were injected with ~100 bacterial cells, whilst insects in Chapter 4 were injected with ~1000 bacterial cells.

Injections into *Manduca sexta*

Newly moulted (Day 0) fifth instar larvae were placed on ice for 5-10 minutes until they were immobilised. The insect was sterilised with 70% ethanol prior to injection of substances. Puncturing the hindmost segment (anterior to the horn) with a disposable 1 mL polycarbonate 30-gauge hypodermic needle, the substance was injected into the haemocoel. Any leaking haemolymph was mopped up and the caterpillars placed back onto diet. The insects were incubated as described above.

Bioassays

Bioassays were done to investigate the effect of treatments on *M. sexta*. Insects were injected as above with the treatments. Each treatment used 10 insects and was repeated 3 times. Because the repeats were highly reproducible, the results of the 3 experiments were combined to give a sample size of 30 insects for each time point. In some experiments, sterile distilled water was used, and in other experiments, endotoxin-free water was used. There was no difference found between these treatments. Mortality was assessed by a failure to respond to poking with a needle.

Bleeding and dissecting *Manduca sexta*

The caterpillars were placed on ice for 5-10 minutes to immobilise them. Next the insects were cleaned with 70% ethanol. The dorsal horn was cut at the tip, and bled into individual prechilled 1.5 mL microcentrifuge tubes. The total amount of haemolymph collected was ~500 µL per insect. Phenylthiocarbamide (PTC) (5 µL) was added to prevent melanisation.

A cut was made with scissors below the dorsal horn and above the anus. Using this hole, the insect was then cut dorsally, taking care not to rupture the gut. The larvae were pinned out and ~100 mg of fat body tissue removed and placed in a 1.5 mL microcentrifuge tube. This was either kept on ice or at -20°C until needed.

RNA extraction

Under fume extraction, 500 µL of TRI Reagent™ (Sigma) was added to the tissue and this was homogenised using a plastic grinder (Sigma) until the tissue was sufficiently ground up (~5 minutes). Then, a further 500 µL of TRI Reagent™ was

added to make a total of 1 mL. This was mixed and centrifuged at 25,000 G for 10 minutes at 4°C. After centrifugation, the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and allowed to stand for 5 minutes. Two hundred micro-litres of chloroform (Sigma) was added to the tube and vortexed until thoroughly mixed (~10 seconds) and then left to stand for 10 minutes at room temperature. After standing, the samples were centrifuged at 25,000 G for 15 minutes at 4°C. The top aqueous phase was transferred to new 1.5 mL microcentrifuge tubes. Next, 500 µL of isopropanol (Sigma) was added to the tube and the sample mixed by inverting several times. The tubes were then left to stand at room temperature for 10 minutes. After standing, the samples were centrifuged at 25,000 G for 10 minutes at 4°C. The supernatants were removed and the pellets washed with 1 mL of 70% ethanol. The samples were centrifuged at 9,500 G for 5 minutes at 4°C. The supernatant was removed and the pellets air-dried for ~10 minutes. The samples were then resuspended in 50 µL of Dimethyl pyrocarbonate (DMPC) treated water and stored at –20°C.

DNase treatment of RNA extractions

The samples were treated with DNase I (Ambion) to remove any DNA present in the tube. 1 µL of DNase I and 2 µL of buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$) was added to each tube and then incubated in a 37°C waterbath for 1 hour. Next the samples were transferred to a heat block and incubated at 75°C for 10 minutes in order to deactivate the enzyme.

Agarose gel electrophoresis

Agarose gel electrophoresis was run on a regular basis to check results of RNA extractions and PCR products. A 1% agarose gel was made by weighing 0.8 g of agarose powder (Invitrogen) in a 100 mL conical flask and adding 80 mL of Tris-acetate-EDTA (TAE) buffer (recipe in Appendix 1). This was heated in a microwave oven to dissolve the powder. After the solution had cooled to ~55°C, 5 µL of ethidium bromide was added, mixed and the solution poured into a gel block and a gel comb inserted. The gel was left to set before being placed in to the gel tank and TAE buffer was used to fill the tank. The comb was removed and samples mixed with 1x loading buffer (Promega) were loaded on to the gel along with DNA and

RNA markers of the appropriate size range (New England BioLabs). The gel was run at 100V for 60 minutes and was then observed under a UV transilluminator.

PCR

Primer design

Clone sequences for each gene were extracted from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The output sequence was pasted into Primer3 (http://frodo.wi.mit.edu/) which selected suitable priming sites. Primer size was specified between 20-24 nucleotides (nt), the melting temperature (Tm) range between 59^{o}C – 61^{o}C and the max complementarity to 5 and Max 3' complementarity to 1 as recommended for RT-PCR primer design (Anon). Resulting primer sequences, corresponding product sizes for each gene are listed in Appendix 2. Primers were synthesised by the company MWG Biotech AG.

RT-PCR

Reverse Transcriptase (RT) - PCR was used to detect mRNA levels of investigated genes. An OneStep RT-PCR kit (Qiagen) was used to perform the RT-PCR following the protocol outlined in the product manual. A master mix of 50 µL total volume containing 0.6 µM of forward and reverse primers, enzyme mix (Omniscript™ RT, Sensiscript™ RT and HotStarTaq® DNA Polymerase), buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 12.5 mM $MgCl_2$, DTT; pH 8.7), dNTPs (10 mM each of dATP, dCTP, dGTP and dTTP) and RNase-free water was used to amplify 2 µg of RNA sample. A PTC-100 thermal controller (MJ Research) was used to control the following thermal profile:

Reverse Transcription	50°C	30 minutes	
Initial PCR Step	95°C	15 minutes	
Denaturation	94°C	30 seconds	
Annealing	50°C	30 seconds	35 cycles
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	

The products were checked by agarose gel electrophoresis. The cDNA sequences of the RT-PCR products were compared to the reference cDNA sequence for the desired gene contained in the NCBI database and found to be correct. The expression of ribosomal protein S3 (rpS3) was used as a control to ensure equal loading of RNA between samples so direct comparisons can be made. All RT-PCRs were carried out using the conditions above. No sample controls and PCRs (No RT, using the same conditions as outlined above) were used to ensure amplification was not due to contamination.

PCR

PCR was used to amplify the inserts from the cloning vector for sequencing and transcription of single stranded RNA. A master mix of 50 µL total volume containing 0.2 µM of forward and reverse primers, *Taq* DNA polymerase, buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton® X-100) $MgCl_2$ (25 mM), dNTPs (10 mM each of dATP, dCTP, dGTP and dTTP) and sterile water was used to amplify 2 µg of DNA. All PCR reagents were sourced from Promega. A PTC-100 thermal controller (MJ Research) was used to control the following thermal profile:

Initial PCR Step	95°C	5 minutes	
Denaturation	95°C	30 seconds	34 cycles
Annealing	50°C	30 seconds	
Extension	72°C	2 minutes	

The products were checked by agarose gel electrophoresis. To ensure that the correct gene had been cloned, the cDNA sequences of the PCR products were verified against the reference cDNA sequence for the desired gene contained in the NCBI database.

qPCR

qPCR was used to detect mRNA levels of investigated genes. A QuantiTect® SYBR® Green RT-PCR kit (Qiagen) was used to perform the qPCR following the protocol outlined in the product manual. A master mix of 25 µL total volume containing 0.6 µM of forward and reverse primers, QuantiTect SYBR Green RT-

PCR Master Mix (HotStarTaq® DNA Polymerase, buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 5 mM $MgCl_2$, pH 8.7), dNTP Mix (dATP, dCTP, dGTP and dTTP/dUTP), SYBR Green I and ROX), RT enzyme mix (Omniscript™ RT and Sensiscript™ RT) and RNase-free water was used to amplify 2 µg of RNA sample. A qPCR DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research) was used to control the following thermal profile:

Reverse Transcription	50°C	30 minutes	
Initial PCR Step	95°C	15 minutes	
Denaturation	94°C	15 seconds	35 cycles
Annealing	50°C	30 seconds	
Extension	72°C	30 seconds	

The qPCR products were subjected to a melting curve analysis following the end of the above profile to verify their specificity and identity. RT-PCR was performed beforehand, and the products sequenced and verified against the NCBI database to ensure primer specificity. No sample and no RT controls were used to ensure that amplification was not due to contaminants. Known amounts of rpS3 single-stranded RNA (ssRNA) were used to generate a standard curve of baseline-subtracted threshold (CT) values, from which absolute concentrations of cDNA contained within samples were calculated.

PCR product purification

A Montage PCR centrifugal filter device (Millipore) was used to purify PCR and RT-PCR products in readiness for sequencing following the protocol supplied. This method of DNA purification uses a regenerated cellulose membrane to filter out the primers and unincorporated nucleotides and dry out the sample. The product is reconstituted with water and recovered by an inverted spin. The products were again checked by agarose gel electrophoresis.

Sequencing

Samples were sent to Qiagen for sequencing. Sanger Cycle sequencing was carried out using a Applied Biosystems Big Dye Terminator Kit 3.1, QIAGEN DyeEx96 Kit for Dye Terminator removal and Applied Biosystems 3730 for capillary electrophoresis and sequence detection. The returned sequences were verified against the NCBI database using software provided by www.geospiza.com/finchtv.

Western blot

Western blots were used to detect protein levels within haemolymph of insects. First, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. The gel plates were first cleaned with detergent, rinsed with distilled water and wiped with 70% ethanol before being assembled in the gel apparatus according to the manufacturer's instructions (Bio-Rad). A 12% acrylamide separating gel (recipe in Appendix 1) was prepared and pipetted between the plates and left to set (~45 minutes). Next, a 5% acrylamide stacking gel was prepared and pipetted between the plates on top of the separating gel, and a gel comb inserted and left to set (~30 minutes). Haemolymph plasma samples were diluted into sample buffer (recipe in Appendix 1), spun at 30,000 G for 2 minutes, heated at 95°C for 5 minutes, and then spun again at 30,000 G for 2 minutes. The gel comb was removed and the wells washed out with distilled water and the running apparatus assembled according to the manufacturer's instructions. Running buffer (recipe in Appendix 1) was added to both the inner and outer reservoirs before samples were loaded into the wells using gel-tips. The apparatus was then run at 120V for 2 hours. The gel was removed and placed in a transfer cassette with a nitrocellulose membrane (Bio-Rad). The samples were then transferred to the nitrocellulose membrane in Towbin buffer (recipe in Appendix 1) at 100V for one hour. The nitrocellulose membrane was then incubated at room temperature for one hour in a blocking buffer (PBS containing 5% skimmed milk powder (Marvel)). The blocking buffer was then discarded and the nitrocellulose membrane incubated at 4°C overnight in an antibody solution (Tween PBS (TPBS) (recipe in Appendix 1) containing 3% skimmed milk powder) with a dilution of the primary antibody specific to the protein of interest. The primary antibody solution was discarded and the nitrocellulose washed twice for ten minutes with TPBS at room temperature. The nitrocellulose membrane was then incubated

with a secondary antibody solution (TPBS containing 3% skimmed milk powder with a 1/10,000 dilution of horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody (Upstate)) at room temperature for 1 hour. The secondary antibody solution was discarded and the nitrocellulose membrane washed twice with PBS for ten minutes. A Visualizer Western Blot Detection kit (Upstate) was used for detection of protein blots following the protocol supplied. After addition of the 'working' solution and incubation for five minutes the nitrocellulose membrane was wrapped in Saran Wrap. Film (Kodak) was timed, exposed to the nitrocellulose membrane in a dark room and then developed.

Production of dsRNA

The cloning and transformation step was performed using a TOPO TA cloning kit (Invitrogen). Two micro-litres of purified RT-PCR product was used in the TOPO cloning reaction, which was carried out using the supplied protocol. The resulting product was used to transform TOP10 competent cells using the Rapid One Shot chemical transformation protocol as printed in the product manual. As indicated in the protocol, the cells were plated out onto LB containing 1.5% agar + ampicillin (concentration 100 µg/ml) plates for selection of the transformants. Colonies were checked for the insert using PCR as outlined above. Colonies with the correctly sized insert were picked with a sterile toothpick and cultured overnight in 5 mL of LB + ampicillin (concentration 100 µg/ml) media at 37^{o}C. A Qiagen Qiaprep spin miniprep kit (Qiagen) was used to extract and purify the plasmids by following the supplied protocol. The products were checked by agarose gel electrophoresis. Inserts were then amplified using PCR. The PCR products from the insert amplification step were used as a template for the synthesis of double-stranded RNA (dsRNA) using the T3 and T7 Megascript kit (Ambion). Four micro-litres of PCR product was used to generate the sense and antisense strands. These were each checked by agarose gel electrophoresis. Next the strands were treated with DNase (Ambion) and then recovered by lithium chloride precipitation according to the supplied protocol. These products were once again run on a 1% agarose gel to determine the approximate concentration of the recovered RNA by inference from band density. Equal amounts of both strands were combined in a 1.5 mL micro-centrifuge tube and placed in a preheated water bath at 70^{o}C and incubated for 15 minutes. After this, the water bath

was switched off and the two strands left to anneal overnight. The product was compared with the two single-stranded RNAs by agarose gel electrophoresis to check whether it had successfully annealed and to determine the concentration of the resulting product.

Fluorescent-activated cell sorting (FACS)

FACS Experiment 1 – Flow cytometry analysis of *Manduca sexta* response to infection with *E. coli* and *P. luminescens* strain TT01.

M. sexta were injected as described above with *P. luminescens* strain TT01, *E. coli*, PBS or left untreated and incubated as described above for 18 hours.

The caterpillars were placed on ice for 5-10 minutes to immobilise them. Next the insects were cleaned with 70% ethanol. The dorsal horn was cut at the tip, and 100 µL of haemolymph bled into individual prechilled 1.5 mL microcentrifuge tubes containing 900 µL anti-coagulant saline (recipe in Appendix 1) and inverted to mix. The samples were centrifuged at 180 G for eight minutes at 4°C. The supernatant (plasma) was removed and the cells re-suspended in 1 mL of Grace's Insect Medium (GIM) (Sigma). Next, 5 µL of Fluorescein isothiocyanate (FITC) labelled Peanut Agglutinin (PNA) was added to each sample, inverted to mix and incubated on ice for 30 minutes. The samples were centrifuged at 180 G for 8 minutes at 4°C and the supernatant removed. The samples were re-suspended in GIM, and were analysed on a BD FACSCanto™ flow cytometer. Both forward scatter (FSC-H) and side scatter (SSC-A) voltages were adjusted to appropriate values to allow analysis. Events smaller than 25,000 units on both scales were not counted. Cells of the appropriate size were analysed for green fluorescence (530 ± 30nm) or not using a 488nm laser. Ten thousand events were recorded for each sample. Results were analysed using the supplied software.

FACS Experiment 2 – Flow cytometry analysis of GFP-*E. coli* and GFP-*P. luminescens* strain TT01 phagocytosis by *Manduca sexta*

M. sexta were injected as described above with Green Fluorescent Protein (GFP) expressing *P. luminescens* strain TT01, GFP-expressing *E.coli*, PBS or left untreated and incubated as described above for 18 hours. GFP-expressing *E. coli* and *P.*

luminescens strain TT01 were obtained from Maria Sanchez-Contreras and Nick Waterfield of the University of Bath. The bacteria were kept and grown as described above in bacterial culture.

The insects were bled as described above in FACS experiment 1. Samples were centrifuged at 180 G for 8 minutes at 4°C. The supernatant (plasma) was removed and the cells re-suspended in GIM. Cell sorting analysis was carried out as described above.

FACS experiment 3 – Flow cytometry analysis of phagocytosis of GFP-*E.coli* by *Manduca sexta* pretreated with bacteria

M. sexta were injected with *P. luminescens* strain TT01, *E.coli*, PBS or left untreated and incubated as described above for 18 hours. Next, all insects were injected with GFP-expressing *E. coli* and incubated at room temperature for 1 hour.

The insects were bled as described above in FACS experiment 1. Samples were centrifuged at 180 G for 8 minutes at 4°C. The supernatant (plasma) was removed and the cells re-suspended in GIM. Cell sorting analysis was carried out as described above.

Confocal microscopy

M. sexta were injected with GFP-expressing *P. luminescens* strain TT01, GFP-expressing *E. coli* or left untreated and incubated for 18 hours.

Cell monolayers were prepared as follows. Insects were bled as described above for FACS analysis. The samples were dropped until they covered a circular coverslip (Fisher) and incubated for 30 minutes to allow cells to attach. 500 µL of 4% para-formaldehyde (Sigma) was added to 'fix' the cells and incubated for five minutes. The monolayers were washed twice with Grace's Insect Medium (GIM). 500 µL of 2% Bovine Serum Albumin (BSA) (Sigma) was added to the monolayers and incubated for 30 minutes. The monolayers were washed twice with GIM. 20 µL of Propidium iodide (Sigma) was added to stain the monolayers and incubated for 15 minutes. The monolayers were washed twice with GIM. The coverslips were then stuck to glass slides (Fisher) with 4% MoWiol (Sigma). The slides were analysed on

a Zeiss LSM510 Confocal microscope using the Argon 488nm and HeNe 633nm lasers. Three insects were used per treatment, and 20-30 cells examined per slide.

Incubation of GFP-expressing *Escherichia coli* in cell-free plasma

Insects were injected as per FACS experiment 1.

Pre-treated insects were bled as normal. The samples were centrifuged at 180 G for eight minutes at 4°C. 0.5 mL of supernatant was removed and placed in a fresh 1.5 mL micro-centrifuge tube. ~500 GFP-expressing *E. coli* cells were added to each cell-free plasma sample, and incubated at room temperature for one hour. 5 µL of cell-free plasma was then plated out on selective plates and incubated overnight at 37°C. Colony forming units were counted the next day.

Chapter 3 – Investigating the role of PGRP in the immune system of *Manduca sexta*

Introduction

As discussed previously in the introduction, in order to mount an effective immune response, an insect needs to able to recognise the presence of non-self within its own body. Microbe-associated molecular patterns (MAMPs), most of which are associated with the bacterial cell envelope, are examples of non-self that are recognised in this way. Examples of MAMPs include peptidoglycan (PGN), lipopolysaccharide (LPS) and flagellin (Samakovlis et al., 1992). The molecular basis of MAMP recognition depends on PRRs produced by the host insect. There are a number of known insect pattern recognition receptors (PRRs) including peptidoglycan recognition protein (PGRP) (Kanost et al., 2004, Yu et al., 2002).

In contrast to the PGRP system in *Drosophila*, little is known about PGRP in *Manduca sexta*. There are two PGRP genes, PGRP-1A and PGRP-1B, but they only differ slightly in their nucleotide sequences. The mature proteins that these two genes produce are identical with only amino acid leader sequences differing (Zhu et al., 2003). The 19 kDa protein shares 54 and 61 % sequence identity to PGRP proteins of fellow lepidopteran insects *B. mori* and *Trichoplusia ni* PGRP. *B. mori* PGRP has been shown to bind to PGN and initiate the PPO cascade (Kanost et al., 2004). However, injection of *M sexta* PGRP into the plasma does not enhance PPO activation in response to *Micrococcus luteus* but does stimulate antibacterial peptide production. The structure of *B. mori* PGRP, like all PGRPs, is similar to that of bacteriophage T7 lysozyme, but lacks key amino acid residues necessary for catalytic activity. This suggests that *M. sexta* is not an amidase but acts as a PRR to stimulate antimicrobial peptide production (Jiang, 2008).

The aim of the work reported in this chapter was to further elucidate the role of PGRP in the immune response of *M. sexta* to challenge with both a benign and pathogenic Gram-negative bacterium. Specifically, I asked:

- What is the time course of increased expression of PGRP mRNA following exposure to Gram-negative bacteria?

- Does RNAi-mediated knockdown of PGRP effect the susceptibility of *M. sexta* to *Photorhabdus luminescens* strain TT01?
- What happens to expression of immune effector genes when PGRP expression is knocked down using RNAi?

Results

PGRP mRNA transcription is induced by *Escherichia coli* and *Photorhabdus luminescens* strain TT01

To determine if the PGRP encoding gene is up-regulated after bacterial infection, RNA was isolated from fat body of *Manduca sexta* 18 hours after challenge with *E. coli* or *P. luminescens* TT01, and the level of PGRP mRNA was determined using RT-PCR.

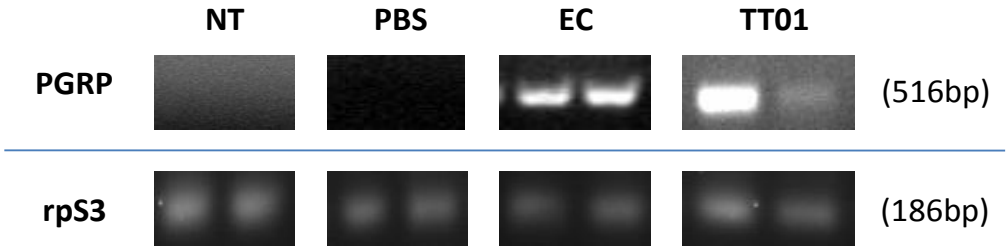

Figure 3.1 – Peptidoglycan recognition protein (PGRP) mRNA expression is induced by both *Escherichia coli* (EC) and *Photorhabdus luminescens* strain TT01 (TT01). Images show RT-PCR products. The untreated (NT) control shows that PGRP mRNA is not present in naive insects, while insects injected with phosphate buffered saline (PBS) show that PGRP mRNA expression is not induced when the insect is wounded with the needle. Each panel shows 2 experimental samples from different insects. This experiment was repeated with a different set of insects and results found to be the same. Expression of a ribosomal protein gene rpS3 was used as a loading control.

As shown by Figure 3.1, both *E. coli* and *P. luminescens* TT01 caused the amount of PGRP mRNA in fat body to increase, while the amount of the control gene (rpS3) did not change. RpS3 was previously used as a loading control by Michael Kanost's research group (Jiang et al., 2004) and also in our papers (Eleftherianos et al., 2006a). The significance of using this gene is that it is constitutively expressed and thus allows us to use it as a loading control to ensure that direct comparisons can be made between different treatments. The extent of the induction of PGRP mRNA was not exactly measured by the RT-PCR technique. Controls of insects injected with

PBS or left untreated had undetectable levels of PGRP mRNA. Nevertheless, it is safe to conclude that the PGRP is strongly induced. It should be noted that the PGRP primers used in these experiments were unable to distinguish between *PGRP-1A* and *PGRP-1B* mRNA sequences found in the NCBI database. Since these sequences are almost identical, and do not differ in the primer regions, the RT-PCR most probably amplifies both mRNAs equally, but this was not checked.

Expression of PGRP protein is induced by *Escherichia coli* and *Photorhabdus luminescens* strain TT01

To determine if the up-regulation of the PGRP mRNA caused by infection with *E. coli* or *P. luminescens* TT01 also results in increased levels of PGRP protein, haemolymph was isolated from *M. sexta* 18 hours after challenge with bacteria, and a Western blot experiment was done to detect expression of PGRP protein using a antibody against PGRP (1/10,000 dilution), a generous gift from Michael Kanost, Kansas State University.

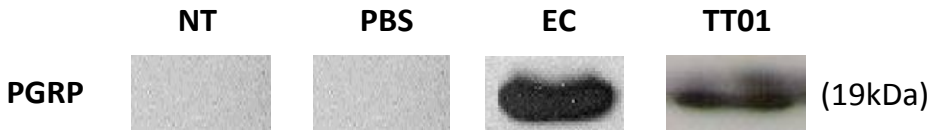

Figure 3.2 – Expression of peptidoglycan recognition protein (PGRP) is induced by both *Escherichia coli (*EC) and *Photorhabdus luminescens* strain TT01 (TT01). Images show bands on a Western blot. An untreated (NT) control shows that PGRP is not present in naive insects. Also PGRP expression is not induced by wounding with a needle as shown by the phosphate buffered saline (PBS) control. This experiment was repeated with a different set of insects and the results found to be the same.

As shown in Figure 3.2, the level of PGRP protein is increased following challenge by both *E. coli* and *P. luminescens* TT01. Control insects that were injected with PBS or left untreated had undetectable levels of PGRP.

The time course of transcription of PGRP mRNA

To study the time course of PGRP mRNA transcription following infection, RNA was isolated from fat body of *M. sexta* at various time-points after challenge with *E. coli* and qPCR was used to determine the mRNA levels of PGRP at these time-points.

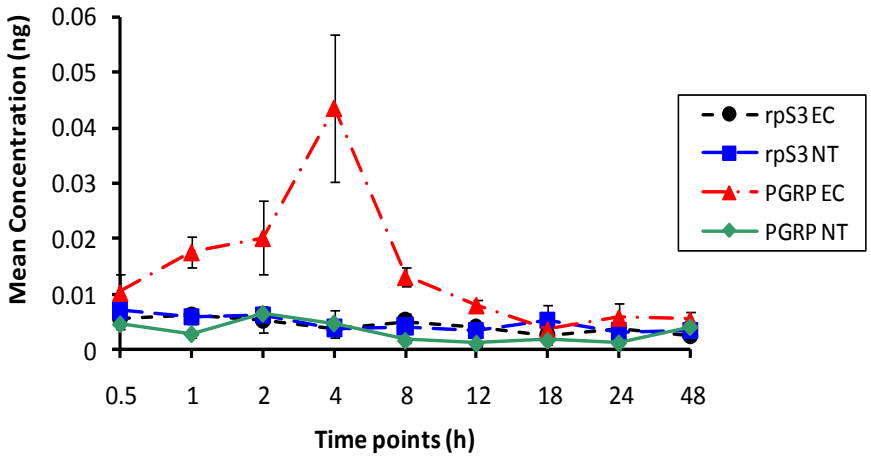

Figure 3.3 – Expression over time of peptidoglycan recognition protein (PGRP) mRNA following challenge with *Escherichia coli* (EC). The graph shows the results from quantitative (real-time) PCR experiments The points show mean values ± standard errors (n=5). The green line represents the untreated (NT) control PGRP mRNA expression. The red line represents PGRP mRNA expression of insects treated with EC. This shows a sharp rise in PGRP expression after infection peaking at 4 hours, then it decreases, falling back to constitutive levels at 18 hours. The rpS3 controls show the background level of mRNA expression.

In this experiment, the transcription of PGRP mRNA peaked at 4 hours following the injection of *E. coli* (Figure 3.3). The response to infection is quick, so that levels of PGRP mRNA doubled only 1 hour after the initial challenge. The response is transient, however, so that elevated levels of PGRP mRNA continue until a peak is seen at 4 hours, but then levels then fall back to constitutive levels by 18 hours. In untreated insects, PGRP mRNA levels remained at a constitutive level throughout the experiment.

Injection with dsRNA for PGRP reduces the level of PGRP mRNA

To determine if treating *M. sexta* with dsRNA for PGRP reduced the levels of PGRP mRNA, RNA was isolated from fat body of *M. sexta* that had first been injected with dsRNA for PGRP then 6 hours later, injected with *E. coli*. The mRNA levels of PGRP were determined using RT-PCR.

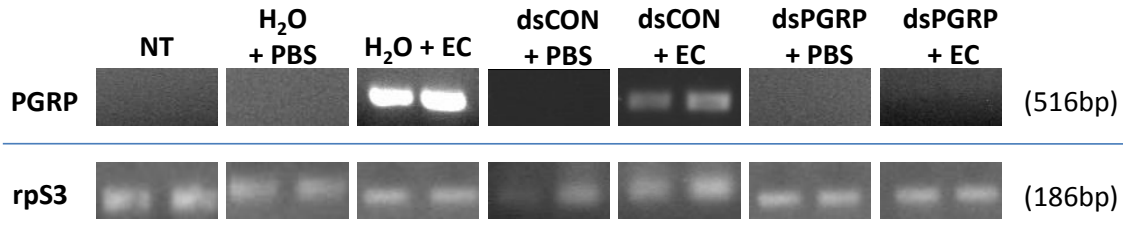

Figure 3.4 – After injection with dsRNA for peptidoglycan recognition protein (dsPGRP), the mRNA expression of PGRP is reduced to a non-detectable level (as assessed by RT-PCR) when the insect is challenged with *Escherichia coli* (EC) six hours after the initial injection. Injection with dsRNA for a control gene (dsCON) from a 'irrevelant' plant gene shows that injection with dsRNA does not induce the immune system (dsCON + PBS) and that dsPGRP is specific to PGRP (dsRNA + EC). As shown previously in Figure 3.1 PGRP mRNA is not present in naive insects (NT) or when the insect is wounded by injection (H_2O + PBS). PGRP mRNA is expressed when the insect is challenged with *E. coli* (H_2O + EC). Each panel shows 2 experimental sample from different insects. This experiment was repeated with a different set of insects and the results found to be the same. Expression of a ribosomal protein gene rpS3 was used as a loading control.

As shown by Figure 3.4, the mRNA levels of PGRP in fat body are reduced to a non-detectable level by treatment with dsRNA for PGRP. Controls of insects treated with water instead of dsRNA showed strong induction of PGRP mRNA when challenged with *E. coli*.

Expression of PGRP protein is reduced with injection of dsRNA for PGRP

To determine if the reduction in PGRP mRNA levels caused by dsRNA for PGRP also results in a reduction in expression of PGRP protein, haemolymph was isolated from *M. sexta* that had first been injected with dsRNA for PGRP then 6 hours later, injected with *E. coli* and was used in a Western blot experiment to detect PGRP protein expression.

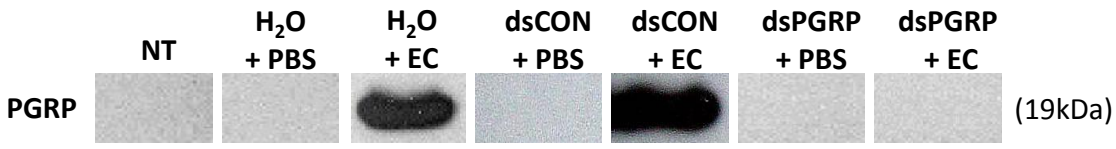

Figure 3.5 – No peptidoglycan recognition protein (PGRP) is produced when the insect is injected with dsRNA for PGRP and challenged with *Escherichia coli* (dsPGRP + EC), whereas when no dsRNA is injected and the caterpillar is challenged with *E. coli* (H_2O + EC), PGRP is induced. Images show bands from a Western blot experiment. As shown previously in Figure 3.2 PGRP is not present in naive insects (NT) or when the insect is wounded by the needle but not challenged with bacteria (H_2O +PBS). The injection of dsRNA from a control does not induce the production of PGRP (dsCON +PBS), and shows that dsPGRP is specific for PGRP (dsCON + EC). This experiment was repeated with a different set of insects and the results were the same.

As shown by Figure 3.5, PGRP protein levels are reduced to a non-detectable level in those insects treated with dsRNA for PGRP. Controls of insects treated with water instead of dsRNA for PGRP showed expression of protein when challenged with *E. coli*.

Insects treated with dsRNA for PGRP are more susceptible to TT01 infection

To investigate the effect of knocking down PGRP expression in *M. sexta*, *P. luminescens* was injected into insects treated with dsRNA for PGRP. Insects were checked every 12 hours over a period of four days (96 hours) for survival.

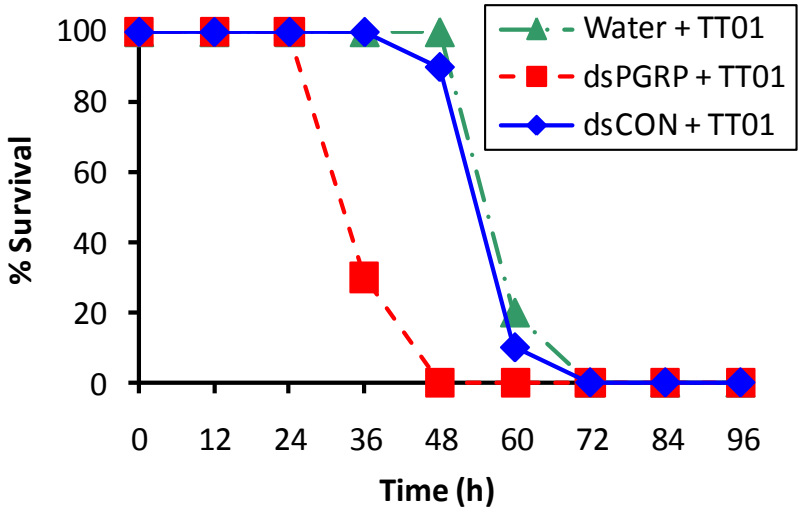

Figure 3.6 - Insects treated with dsRNA for peptidoglycan recognition protein (dsPGRP) are more susceptible to TT01 infection. The graph shows the percentage of insects surviving each treatment. Ten insects were used for each treatment and this experiment was repeated 3 times. All insects treated with dsPGRP + TT01 were killed 12-24 hours before a normal infection (Water + TT01). A control treatment (dsCON + TT01) shows that this is not just a general response to being injected with dsRNA as it is specific (dsCON) for a plant gene encoding a Catalase enzyme.

As shown by Figure 3.6, insects that have been treated with dsRNA for PGRP are killed approximately 24 hours before those insects that were treated with a control dsRNA or water. This indicates that PGRP might have a strong role in the defence of *M. sexta* against pathogen attack.

Injecting with dsRNA for PGRP abolishes the priming effect of pre-immunising *Manduca sexta* with *Escherichia coli*

To determine if knocking down the expression of PGRP had an effect on the priming of *M. sexta* with *E. coli* against *P. luminescens*, insects were treated with dsRNA for PGRP. Six hours later they were injected with *E. coli*. Then, 18 hours after the *E. coli* injection, *P. luminescens* was injected. Insects were checked for survival every three hours for the first 24 hours after the final injection and every 12 hours afterwards until two days (48 hours) had passed.

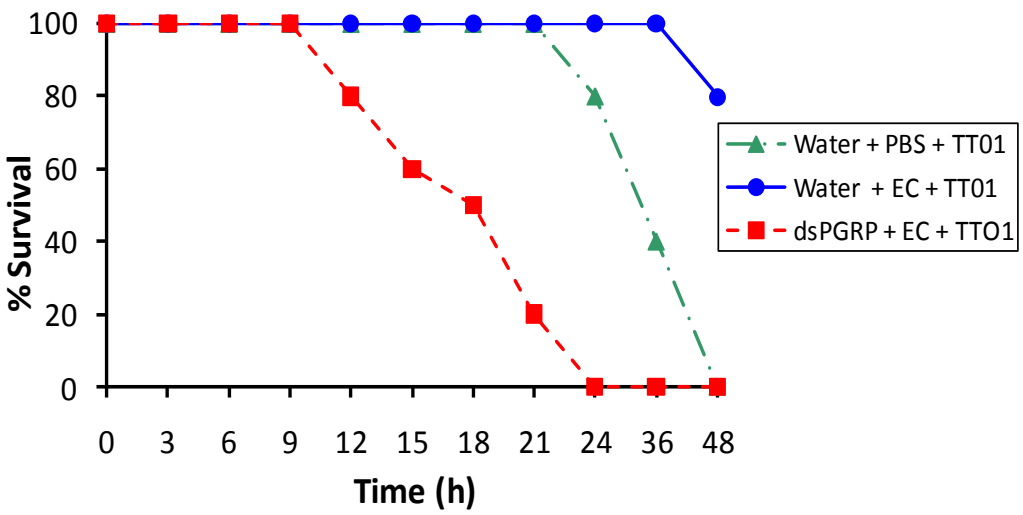

Figure 3.7 – Injecting with dsRNA for peptidoglycan recognition protein (dsPGRP) abolishes the priming effect of pre-immunising *Manduca sexta* with *Escherichia coli*. The graph shows the percentage of insects surviving following each treatment. Ten insects were used in each treatment, and the experiment was repeated 3 times. Insects given TT01 (dsPGRP + EC + TT01) died 12-24 hours before those not given the RNAi treatment (Water + PBS + TT01). If the caterpillar is not treated with dsPGRP then pre-immunising with *E. coli* reduces the susceptibility of *M. sexta* to TT01.

As shown by Figure 3.7, insects treated with dsRNA for PGRP, injected with *E. coli*, then *P. luminescens* are killed within 24 hours of the final injection. In contrast, controls of insects treated with water instead of dsRNA, injected with *E. coli*, then *P. luminescens*, mostly survived with approximately one-fifth being killed. Those insects treated with water, injected with PBS instead of *E. coli*, then *P. luminescens*, were killed within 48 hours.

RNAi knock-down of PGRP reduces expression of anti-microbial responses

To investigate the effect of knocking down the expression of PGRP has on anti-microbial responses, RNA was isolated from fat body of *M. sexta* that had previously been treated with dsRNA for PGRP, then after 6 hours, injected with *E. coli*. RT-PCR was used to determine the mRNA levels of various anti-microbial responses.

First, primers for Attacin were used. Attacin is an anti-microbial peptide.

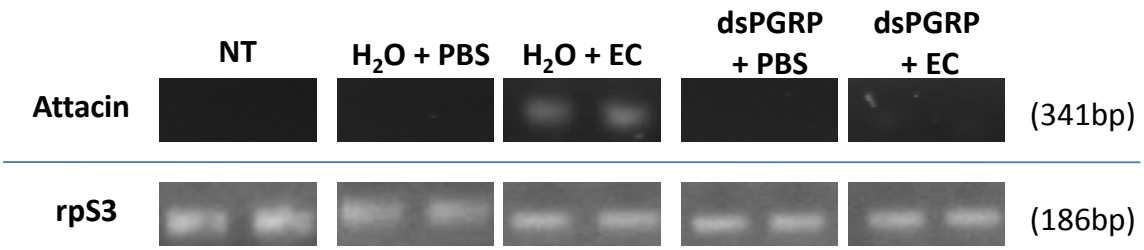

Figure 3.8 – RNAi knock-down of peptidoglycan recognition protein (PGRP) prevents induced expression of the anti-microbial peptide: Attacin. The panels show bands from an RT-PCR experiment. Attacin mRNA expression is induced when *Manduca sexta* is challenged with *Escherichia coli* (H_2O + EC), whereas in naive insects (NT) Attacin is not present. Wounding with a needle also does not induce Attacin mRNA expression (H_2O + PBS). If the caterpillar is treated with dsRNA for PGRP before being challenged with *E. coli* then Attacin mRNA expression is not induced to a detectable level (dsPGRP + EC). Each panel shows 2 experimental sample from different insects. This experiment was not repeated. Expression of a ribosomal protein gene rpS3 was used as a loading control.

As shown by Figure 3.8, Attacin mRNA levels in fat body are increased when *M. sexta* is challenged by *E. coli*, but controls of insects left untreated or injected with PBS had undetectable levels of Attacin mRNA. Attacin mRNA levels in fat body were reduced to non-detectable levels in those insects treated with dsRNA for PGRP.

Next, primers for Moricin were used. Moricin is also an anti-microbial peptide.

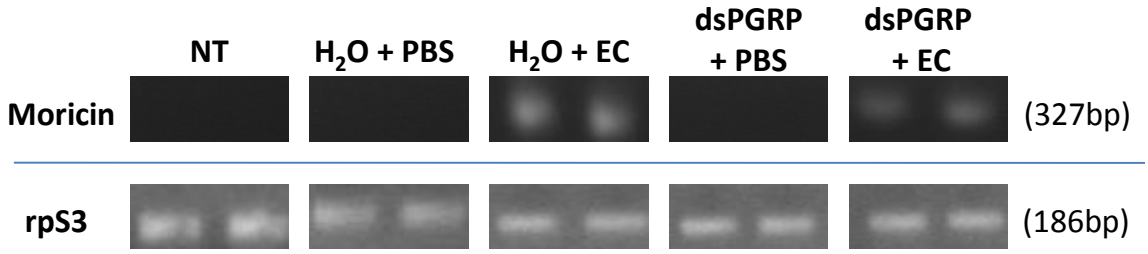

Figure 3.9 – RNAi knock-down of peptidoglycan recognition protein (PGRP) reduces induced expression of the anti-microbial peptide: Moricin. The panels show bands from an RT-PCR experiment. Moricin mRNA expression is induced when *Manduca sexta* is challenged with *Escherichia coli* (H_2O + EC), whereas in naive insects (NT) Moricin is not present. Wounding with a needle also does not induce Moricin mRNA expression (H_2O + PBS). If the caterpillar is treated with dsRNA for PGRP before being challenged with *E. coli* then Moricin mRNA expression is reduced (dsPGRP + EC). Each panel shows 2 experimental sample from different insects. This experiment was not repeated. Expression of a ribosomal protein gene rpS3 was used as a loading control.

As shown by Figure 3.9, Moricin mRNA levels in fat body are increased when *M. sexta* is challenged by *E. coli*, but controls of insects left untreated or injected with PBS had undetectable levels of Moricin mRNA. Moricin mRNA levels in fat body were reduced to barely detectable levels in those insects treated with dsRNA for PGRP.

Finally, primers for Pro-Phenoloxidase were used. Pro-Phenoloxidase is the inactive substrate for Phenoloxidase, which has anti-microbial properties.

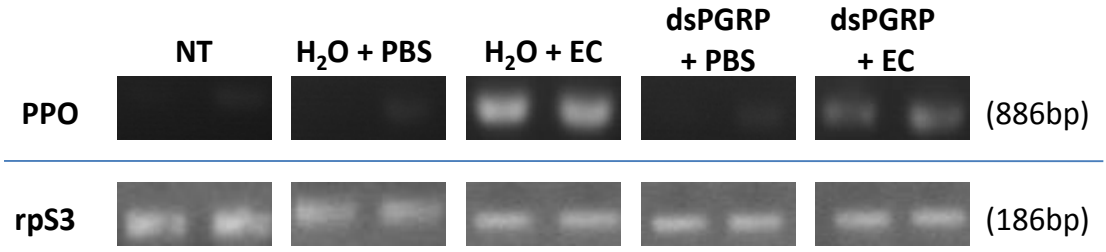

Figure 3.10 – RNAi knock-down of peptidoglycan recognition protein (PGRP) reduces induced expression of Pro-phenoloxidase (PPO). The panels show bands from an RT-PCR experiment. PPO mRNA expression is induced when *Manduca sexta* is challenged with *Escherichia coli* (H_2O + EC), whereas in naive insects (NT) PPO is not present. Wounding with a needle also does not induce PPO mRNA expression (H_2O + PBS). If the caterpillar is treated with dsRNA for PGRP before being challenged with *E. coli* then PPO mRNA expression is reduced (dsPGRP + EC). Each panel shows 2 experimental sample from different insects. This experiment was not repeated.Expression of a ribosomal protein gene rpS3 was used as a loading control.

As shown by Figure 3.10, Pro-Phenoloxidase mRNA levels in fat body are increased when *M. sexta* is challenged by *E. coli*, but controls of insects left untreated or injected with PBS had undetectable levels of Pro-Phenoloxidase mRNA. Pro-Phenoloxidase mRNA levels in fat body were reduced to barely detectable levels in those insects treated with dsRNA for PGRP.

Discussion

The main findings of the work reported in this Chapter were as follows:

- qPCR experiments showed that the level of PGRP mRNA increased soon after bacterial challenge, with a peak seen at 4 hours after infection. This then fell back to constitutive levels at 18 hours.
- RNAi was successfully used to knock down the level of PGRP mRNA in fat body of insects (both unchallenged insects and those challenged with *E. coli*).
- This experimental knock down of PGRP resulted in the affected insects being more susceptible to *P. luminescens*, and even abolishing the priming effect of previously injecting *E. coli*.
- The anti-microbial effectors: Attacin, Moricin and PPO mRNAs were downregulated after the PGRP knock down suggesting that PGRP has a role in up-regulating the expression of the genes.

The need for insects to detect a microbial challenge is very important and here *M. sexta* proves to be no exception to the rule. Insects and other animals use PRRs to detect MAMPs and initiate the immune response, and PGRP is one such PRR used to detect peptidoglycan, a MAMP present in almost all bacteria (Lemaitre and Hoffmann, 2007). Here, it was found that both PGRP mRNA and protein are upregulated in *M. sexta* in response to challenge from both non-pathogenic (*E. coli*) and pathogenic (*P. luminescens*) Gram-negative bacteria (Figure 3.1, Figure 3.2). Furthermore, the response to *E. coli* is quick and transient (Figure 3.3) apparently peaking at four hours after infection, although it should be noted that actual peak could have occurred anywhere between two and eight hours. mRNA expression of PGRP falls back to 'normal' constitutive levels by 18 hours after infection. It is not known what causes the decrease in PGRP mRNA expression; it is possible that the

decrease is due to degradation of the original signal, or perhaps due to a negative feedback system being activated. The fall back to constitutive levels by 18 hours seen in the qPCR experiments (Figure 3.3) is not entirely consistent with the results of the RT-PCR experiments shown in Figure 3.1 as the latter suggest that PGRP mRNA levels remained higher than constitutive levels even after 18h. The two different types of experiments were not conducted concurrently, and it is possible that there were slight differences between the experimental protocols, or that the biological condition of the insects had changed between the two different sets of treatments.

As mentioned above, the injection into *M. sexta* of the pathogenic Gram-negative bacterium *P. luminescens* strain TT01 also resulted in upregulation of PGRP at both mRNA and protein levels, showing that *M. sexta* is able to recognise the presence of this pathogen. Ultimately though, this recognition is unable to prevent *P. luminescens* strain TT01 killing the insect (Figure 3.6). However, if the insect is challenged with *E. coli* prior to infection with *P. luminescens* strain TT01, it is rendered less susceptible to P. luminescens, and as a result the death rate due to the second infection is reduced (Figure 3.7). To investigate the mechanism of this pre-immunisation effect, RNAi was successfully used to knock down the level of PGRP mRNA and as a consequence of this, expression of PGRP protein (Figure 3.4, Figure 3.5). The effect of knocking down PGRP in insects challenged with *P. luminescens* strain TT01 was to increase its susceptibility to the pathogen (Figure 3.6). The knock down effect in those insects pre-immunised with *E. coli* not only abolished the protective effect induced by pre-treatment with *E. coli,* but actually rendered the insects even more susceptible to a *P. luminescens* strain TT01 infection than the insects that had not received the *E. coli* treatment (Figure 3.7). A certain level of redundancy in the immune response might have been expected, similar to the case in *Drosophila*, where genetic ablation of both PGRP-LC and –LE (both of which detect DAP-type peptidoglycan) is required (Lemaitre and Hoffmann, 2007), but the RNAi knock down experiment described here shows that the single PGRP in *M. sexta* plays an important if not essential role in the insect's the immune responses.

It has been suggested that *M. sexta* PGRP has no role in the PPO cascade (Kanost et al., 2004) but that the protein does have a role in the production of antimicrobial peptides. In the present work, I found that RNAi knock down of PGRP resulted in

reduced mRNA levels of Attacin, Moricin and PPO, three important antimicrobial effectors (Figure 3.8, Figure 3.9, and Figure 3.10) that are upregulated in response to challenge with *E. coli*. Unfortunately, further investigation of the effect of PGRP knock down on the expression of other antimicrobial effectors was unsuccessful due to an inability to repeat the RNAi knock down of PGRP. (This problem is discussed in the final chapter). Individual knock down of Attacin or Moricin by RNAi did increase the susceptibility of *M. sexta* to *P. luminescens* strain TT01 although this effect was much less pronounced than the knock down of PGRP (Eleftherianos et al., 2006a). These antimicrobial peptides defend the insect by attacking bacteria present in the haemolymph plasma. Growth experiments of *P. luminescens* strain TT01 in cell-free haemolymph taken from insects treated with dsPGRP show that the knock down has a significant effect on such induced humoral responses (Eleftherianos et al., 2006a). Haemolymph from control insects pre-treated with *E. coli* did not support the growth of P. *luminescens*, while haemolymph from PGRP knock down insects pre-treated with E. coli was able to support prolific growth of *Photorhabdus* (Eleftherianos et al., 2006a). Furthermore, this result is consistent with an experiment that is reported in Chapter 5 (see Figure 5.14). When *E. coli* cells expressing the green fluorescent protein (GFP) were exposed to haemocytes from insects pre-treated with E. coli, virtually all the bacterial cells were killed. This did not occur when haemocytes from insects that had not been pre-treated in this way.

It is apparent that PGRP plays a crucial (although sometimes ultimately futile) role in the immune defence of *M. sexta*, being required for the upregulation of mRNA levels of Attacin, Moricin, PPO and possibly other antimicrobial effectors. The presence of these effectors within the haemolymph plasma is largely responsible for the ability of pre-immunised *M. sexta* to defend itself from *P. luminescens* strain TT01. The cellular mechanism by which PGRP is involved in regulation of expression of these antimicrobial factors is however not known.

Chapter 4 – The *exbD* and *yfeABCD* genes are needed for virulence in *Photorhabdus luminescens* strain TT01

Introduction

Iron is an essential element for life due to its many roles within many organisms. Animals, microbes and plants all require iron for many biological processes such as DNA synthesis, photosynthesis, electron transport and activation of oxygen (Andrews et al., 2003). However, in the presence of oxygen, iron catalyses the formation of hydroxyl radicals, which are damaging to the host organism. It is therefore essential for organisms to tightly regulate the availability of iron. Organisms manage this by sequestering iron into specific carrier proteins.

Iron exists in one of two oxidative states; the oxidised Fe^{3+} ferric state or the reduced Fe^{2+} ferrous state. The abundance of either form depends much upon its current environment; ferric iron is more common in aerobic inorganic environments, whereas ferrous iron is more in anaerobic or reducing conditions (Andrews et al., 2003). Ferric iron is also extremely insoluble and therefore of limited availability to many organisms, in contrast, ferrous iron is quite soluble.

Bacteria, like most organisms have an absolute requirement for iron, although there are a few species that lack this requirement, including *Borrelia burgdorferi* and *Treponema palladium*, two obligatory intracellular pathogens (Wandersman and Delepelaire, 2004). However, bacteria generally find themselves in an environment whereby the iron is insoluble or not freely available. To counter this, bacteria have several mechanisms designed to obtain iron from its environment. These rely on cell surface proteins to recognise and transport iron or iron-containing molecules across the membrane. The mechanisms used to obtain iron can be direct or indirect (Law, 2002). Direct mechanisms involve an interaction between the bacteria and the iron source. Indirect mechanisms involve iron chelators released from the bacteria to scavenge for iron sources. These then return to the bacteria and are transported across the membrane.

Direct sources of iron include free iron, transferrin, ferritin, heme and albumin. If iron is present in its ferrous state then it is easily transported across the membrane by a group of ABC permeases (Koster, 2001). These ABC permeases are generally responsible for the transport of iron-containing molecules (Perry et al., 2007). *feo* and *yfe* systems are responsible for ferrous iron uptake in *Photorhabdus luminescens* strain TT01 and other bacteria. The *feo* system has been proved to be essential for iron acquisition in the stomach and intestines of mammals by both *Salmonella enterica* and *Helicobacter pylori*. Furthermore the loss of *feo* A or B in *Yersinia pestis* results in a loss of iron acquisition activity (Perry et al., 2007). Mutants lacking both *yfeAB* and *feoB* were also unable to grow in mice macrophages. In addition to the transport of ferrous iron, the *yfe* system is also responsible for the transport of manganese in *Y. pestis*. An *yfeAB* mutant shows growth defects when grown on restricted iron media, which was alleviated with the addition of iron. In addition the *yfeAB* mutant shows reduced virulence in mice where it is thought that *yfe* plays a role in the latter stages of infection (Perry et al., 2007).

Indirect sources of iron come from siderophores and hemophores. Siderophores are compounds, generally low in molecular weight, that chelate iron with a very high affinity (above 10^{30} M^{-1}, transferrin has a affinity for Fe^{3+} of ~ 10^{20} M^{-1}). Hemophores have only been found in Gram-negative bacteria and they acquire heme for the bacteria. Although there has been over 500 different siderophores described, most of them have a similar structure; a peptide backbone consisting of several non-protein amino acids, with the iron ligation groups attached to this (Wandersman and Delepelaire, 2004). The diversity of siderophores is mainly determined by which ligand groups are present. The siderophores are excreted from the cell by an unknown mechanism to search for iron. Ferri-siderophores (siderophores that have managed to pick up an iron molecule) are recognised by outer-membrane receptors in Gram-negative bacteria and by membrane-anchored binding proteins in Gram-positive bacteria. The ferri-siderophores are transferred across the cytosolic membrane by ABC permeases into the cytosol. Gram-negative bacteria first have to transport the ferri-siderophore from the outer-membrane to the cytosolic membrane. This transport is mediated by the TonB complex, which is needed to transduce the energy required for such a process. The TonB complex is made up of three proteins; TonB, ExbB and ExbD. Many studies have proved how important this complex is to

many species of bacteria. Loss of TonB has been shown to result in a loss of virulence for a number of pathogens, including *Pseudomonas aeruginosa*, *Haemophilus influenza* and *Photorhabdus temperata* strain K122. A *P. temperata* K122 mutant lacking *exbD* had much reduced virulence against the Greater Waxmoth *Galleria mellonella* and an inability to grow in iron-restricted media (Andrews et al., 2003).

By contrast, many animals including insects rely on transferrins to pick up free iron. Vertebrate transferrins have been found in blood (serum transferrin); milk, tears and extracellular fluids (lactoferrin); and in egg whites (ovotransferrin) (Andrews et al., 2003). These proteins, though found in different sites, share a close structural relationship. Each is an 80 kDa glycoprotein, which contains two ferric-binding lobes, probably due to gene duplication. On the other hand, although most insect transferrins have only one ferric-binding site, two *Drosophila* transferrins are predicted to two potential iron-binding domains, and a transferrin found in *Blaberus dicoidalis* has been shown to bind two Fe^{3+} ions (Law, 2002). The first insect transferrin to be characterised was that of *M. sexta* and studies using radioactive iron as a tracer showed that it rapidly binds free iron in the haemolymph and transfers it to fat body (Law, 2002). Some of this iron later appears back in the haemolymph but this time bound to ferritin. The mechanisms surrounding this transfer are unclear; the lack of homologous vertebrate transferrin receptors in *Drosophila* suggests that either a different type of receptor or a different transport mechanism is used. Insect transferrin has been shown to be up-regulated in response to microbial challenge in *D. melanogaster*, *Bombyx mori* and the mulberry longicorn beetle, *Apriona germari*, suggesting that transferrin has a role in the immune response (Ong et al., 2006). The promoter region for the transferrin gene of *D. melanogaster* contains binding sites for NF-κB-like transcription factors involved in the immune response. There has been no anti-microbial activity demonstrated by transferrin, so it is suggested that its role within the immune response is to simply withhold iron from the invading bacteria.

Ferritins are proteins used for the storage of iron. Twenty-four subunits make up the structure of ferritin, which essentially consists of a shell surrounding a cavity in which multiple ferric ions are stored. The subunits in insects are made up of homologues to the heavy and light subunits that make up ferritin proteins in

vertebrates and are known as heavy chain homologue (HCH) or H-type and light chain homologue (LCH) or non-H-type respectively (Duchaud et al., 2003). Despite its name, LCH is actually a larger protein than HCH, and the former is so-called because of a lack of ferrioxidase centre residues. Up-regulation of ferritin mostly occurs as a response to the presence in body fluids of excess iron, whereas in response to infection, there is very little change in expression. However, the secretion of ferritin into haemolymph was shown to be up-regulated in response to infection in *D. melanogaster*. Similar to the transferrin gene in the same organism, the ferritin gene also has a NF-κB-transcription factor binding site. This suggests that unlike most other animals, ferritin may play a role in the immune responses of *Drosophila*.

The published genome of *P. luminescens* strain TT01 reveals that this organism possesses the largest number of genes encoding iron and iron-containing molecules of any bacterium, suggesting that the acquisition of iron is very important (Goodrich-Blair and Clarke, 2007). It could also be an adaptation of its lifestyle as it infects many different insects, as well as living in symbiosis with a nematode. A selection of iron-uptake and iron-storage knock-out *P. luminescens* strain TT01 mutants were kindly donated to me by Robert Watson and David Clarke for the purposes of this chapter. The genes that have been knocked out in these mutants and their function are outlined in Table 4.1.

Class	Gene	Function
Transport	*exbD*	Component of the TonB complex
	feoAB	Uptake mechanism of ferrous (Fe^{2+}) iron
	yfeABCD	Uptake mechanism of ferrous (Fe^{2+}) iron
	plu3613	Homology to a putative heme binding protein in *Yersinia pestis*
Storage	*ftnA*	Ferritin
	ppxAB	Photopexins
	plu4231	High similarity to *ppxAB*

Table 4.1 – *Photorhabdus luminescens* strain TT01 genes and their function

These mutants were created using a directed knock-out against the gene of interest. PCR was used to amplify 600bp regions upstream and downstream of the gene of interest with a complementary tail added to the region where the gene should be. These tails will then bind the fragments together creating a knock-out fragment. This knock-out fragment is then cloned into a plasmid. The plasmid is then transferred into *P. luminescens* strain TT01 by conjugation and exconjugants selected by growth on antibiotic-containing agar plates. Full details of the process can be found in (Watson, 2007).

The antibiotic parent strain that was used to make these mutants is a naturally-occuring Rifamycin resistant mutant. Rifamycin acts upon RNA polymerase and prevents RNA synthesis. This will affect many systems and one of the most apparent is that it suffers from decreased virulence when compared to the wild-type. However, comparing knock-out mutants based upon this Rif to the wild-type would not ensure

a fair comparison, so despite the decreased virulence, a Rif –resistant mutant was used as the baseline for comparison of the knock-out mutants.

The aim of this chapter is to further elucidate the role of transferrin and ferritin in the immune response of *M. sexta* and also to investigate the role of iron uptake and storage on the pathogenesis of *P. luminescens* strain TT01. Specifically, I asked:

- Is transferrin and ferritin expression up-regulated in *M. sexta* following an immune challenge with *E. coli*?
- Is there a loss of pathogenicity in iron knock-out mutants of *P. luminescens* strain TT01 against *M. sexta*?
- Can this loss of pathogenicity be reversed using extra iron or RNAi?

Results

Transferrin but not ferritin is up-regulated following infection with *Escherichia coli*

To determine if Transferrin and Ferritin encoding genes are up-regulated following bacterial infection, RNA was isolated from fat body of *Manduca sexta* 18 hours after injection with *E. coli*, and the level of mRNA was determined using RT-PCR.

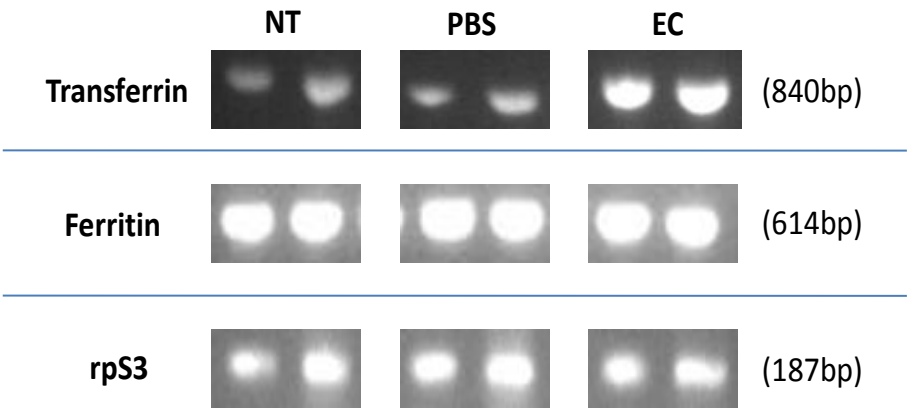

Figure 4.1 – Transferrin but not Ferritin is up-regulated when *Manduca sexta* is injected with *Escherichia coli* (EC). The panels show RT-PCR bands from pairs of similarly – treated insects. Insect challenged with *E. coli* show clear elevation of Transferrin mRNA compared to the constitutive levels that can be seen in the untreated (NT) controls. This doesn't happen when the insect is wounded with a needle (PBS). On the other hand, the mRNA levels of Ferritin don't change upon challenge with *E. coli* or wounding with a needle (PBS) from the untreated control. This experiment was repeated with a different set of insects and the results found to be the same. rpS3 mRNA levels are included as a loading control.

As shown by Figure 4.1, transcription of Transferrin but not Ferritin mRNA in fat body is increased from the constitutive levels seen in the untreated insects following an infection with *E. coli*. Controls of insects injected with PBS showed no change in the level of mRNA in either gene from untreated insects. The mRNA levels of a control gene (rpS3) remained constant. The extent of changes in mRNA levels cannot be exactly measured by the RT-PCR technique, but it is clear from the differences in band intensity that there is up-regulation of the Transferrin gene and no change in Ferritin transcription.

Time-course of Transferrin and Ferritin protein expression

To determine if the up-regulation of the Transferrin encoding gene caused by infection with *E. coli* resulted in an increase in expression of Transferrin protein and if there was any change in Ferritin protein expression as a result of *E. coli* infection, *M. sexta* haemolymph was isolated at various time-points after injection with *E. coli* and a Western blot experiment was used to detect expression of Transferrin and Ferritin proteins using antibodies against Transferrin (1/4,000 dilution) and Ferritin (1/10,000 dilution), generous gifts from John Law and Joy Winzerling, University of Arizona.

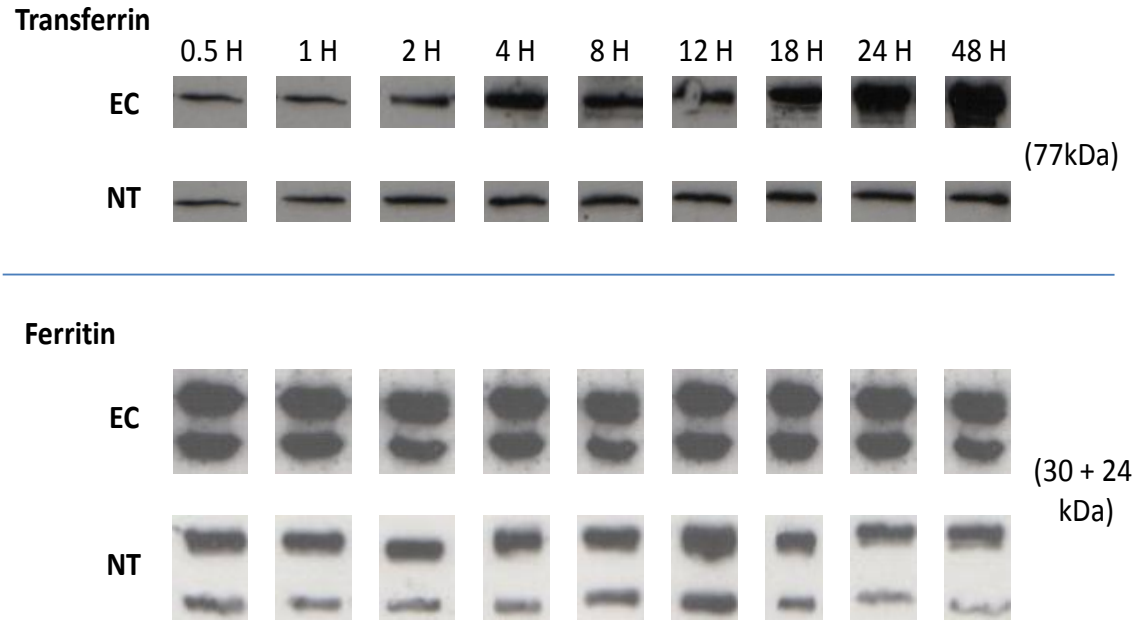

Figure 4.2 – Transferrin protein but not Ferritin is increased when *Manduca sexta* is exposed to *Escherichia coli* (EC) over a time-course of 48 hours (H). Images show bands from a Western blot. The level of Transferrin protein is clearly increased compared to an untreated control (NT). The level of Ferritin remains constant over the time-course in both *E. coli* challenged and untreated insects. This experiment was repeated with a different set of insects and the results found to be the same.

As shown by Figure 4.2, Transferrin protein levels have increased by 18 hours after infection with *E. coli* and continue to increase for at least another 30 hours. By contrast, Ferritin protein levels remain at a constitutive level over the time-course. Controls of untreated insects show no increase from the constitutive levels of either gene.

Survival curve of *Manduca sexta* against *Photorhabdus luminescens* strain TT01 mutants

To determine the effect on pathogenicity of an iron-uptake deficient *P. luminescens* strain TT01 mutant caused by gene knock-out, *M. sexta* was injected with 12 different knock-out mutants featuring 7 genes with roles in iron-uptake. Insects were checked for mortality once every 24 hours until 168 hours (7 days) after the initial injection.

The first mutant to be injected was an *exbD* knock-out. This is a membrane bound protein which forms a complex with *TonB* and *exbB* and is essential for ferric ion uptake in bacteria.

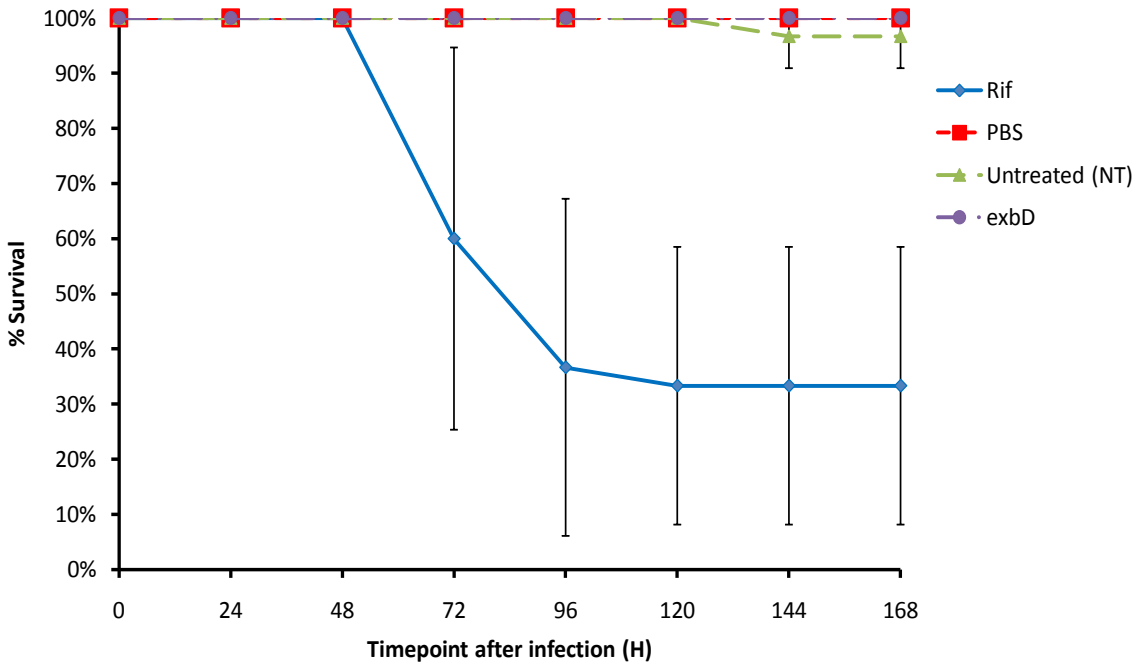

Figure 4.3 - Survival curve of *Manduca sexta* against the *exbD* mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The 100% survival rate shows that *exbD* is unable to kill the caterpillar. The parent strain TT01 *Rif* is able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.3, the *exbD* knock-out mutant is unable to kill *M. sexta*. The parent strain *Rif* is able to kill approximately two-thirds of the insects that it infects. Therefore it is safe to assume that the *exbD* protein has a strong role in the pathogenicity of *P. luminescens* strain TT01. It should be noted that the *Rif* mutant strain of *P. luminescens* used in these experiments was less virulent than the parent wild type TT01 strain used in previous experiments reported in this thesis. For the

number of bacteria injected, the infected insects would have been expected to die more quickly and in greater numbers when given TT01 than was observed in those insects given the *Rif* mutant. This point is discussed further in the Discussion section of this Chapter.

The second mutant to be injected was a *feoAB* knock-out. This is an integral membrane protein that has a role in iron (II) up-take.

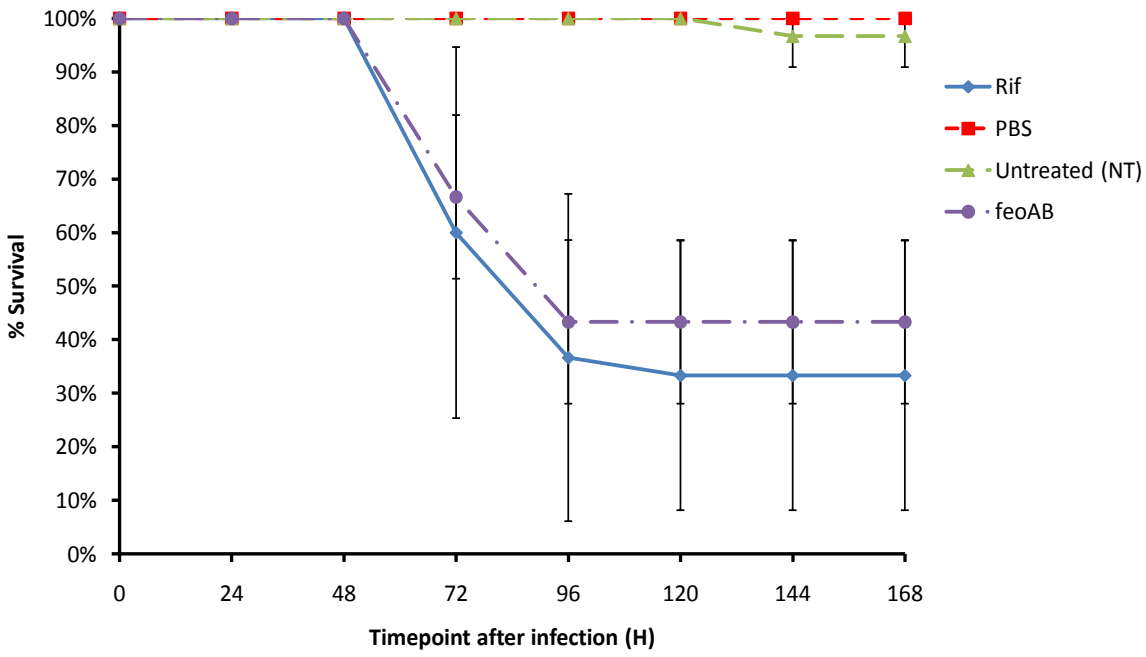

Figure 4.4 - Survival curve of *Manduca sexta* against the *feoAB* mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 Hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. *feoAB* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.4, the *feoAB* knock-out mutant is able to kill *M. sexta* with similar efficacy to the parent strain *Rif*, which was able to kill two-thirds of the insects. This indicates that *feoAB* does not have an essential role in pathogenicity of *P. luminescens* strain TT01.

The next mutant to be injected was an *yfeABCD* knock-out. This protein has a role in transporting iron (II) and Manganese into the cell.

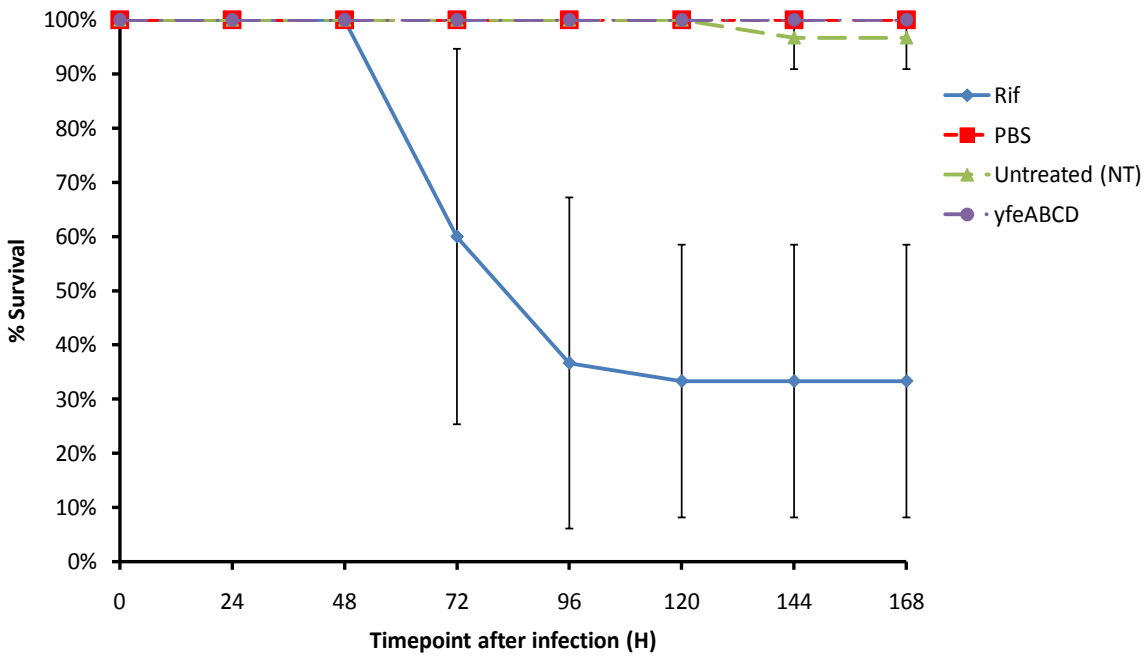

Figure 4.5 - Survival curve of *Manduca sexta* against the *yfeABCD* mutant of *Photorhabdus luminescens* strain TT01. over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The 100% survival rate shows that *yfeABCD* is unable to kill the caterpillar. The parent strain TT01 *Rif* is able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.5, the *yfeABCD* knock-out mutant is unable to kill *M. sexta*. The parent strain *Rif* is able to kill approximately two-thirds of the insects that it infects. Therefore it is safe to assume that the *yfeABCD* protein has a strong role in the pathogenicity of *P. luminescens* strain TT01.

The next mutant to be injected was an *exbD feoAB* double knock-out. *exbD* is a membrane bound protein which forms a complex with *TonB* and *exbB* and is essential for ferric ion up-take in bacteria. *feoAB* is an integral membrane protein that has a role in iron (II) up-take.

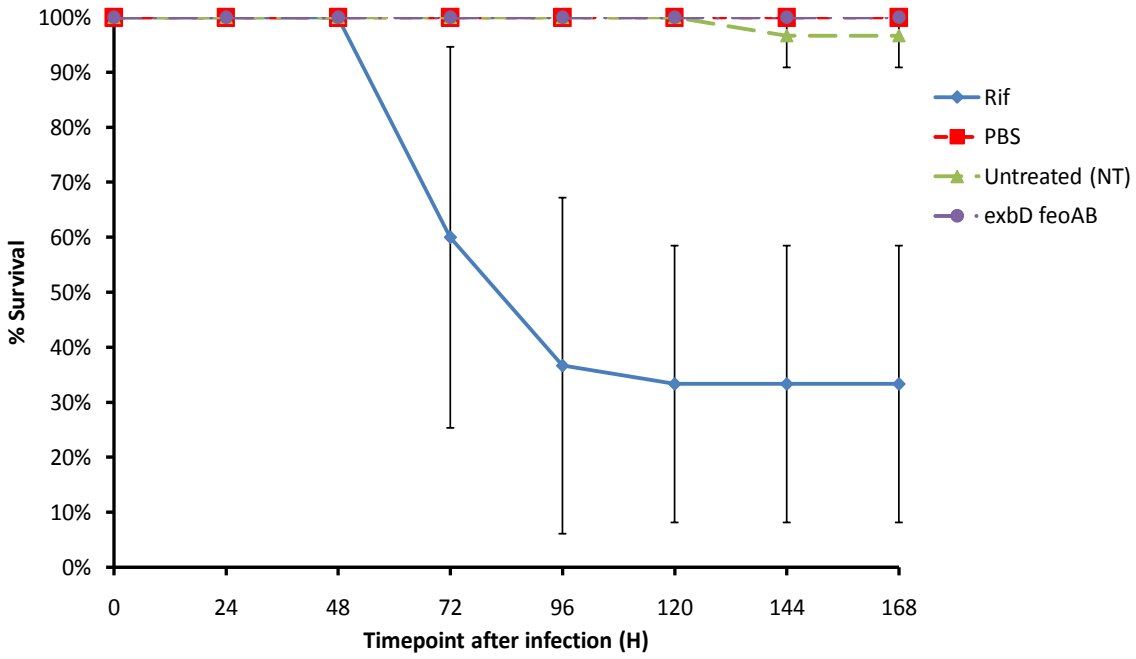

Figure 4.6 - Survival curve of *Manduca sexta* against the *exbD feoAB* double mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The 100% survival rate shows that *exbD feoAB* is unable to kill the caterpillar. The parent strain TT01 *Rif* is able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.6, the *exbD feoAB* double knock-out mutant is unable to kill *M. sexta*. The parent strain *Rif* is able to kill approximately two-thirds of the insects that it infects. This loss in pathogenicity is probably due to the *exbD* knock-out rather than the loss of both genes because as shown previously in Figure 4.3, the *exbD* single knock-out is unable to kill any insects. This indicates that *exbD* has a strong role in the pathogenicity of *P. luminescens* strain TT01, and is an independent confirmation of the result seen in Fig. 4.3.

The next mutant to be injected was an *exbD yfeABCD* double knock-out. *exbD* is a membrane bound protein which forms a complex with *TonB* and *exbB* and is essential for ferric ion up-take in bacteria. *yfeABCD* has a role in transporting iron (II) and Manganese into the cell.

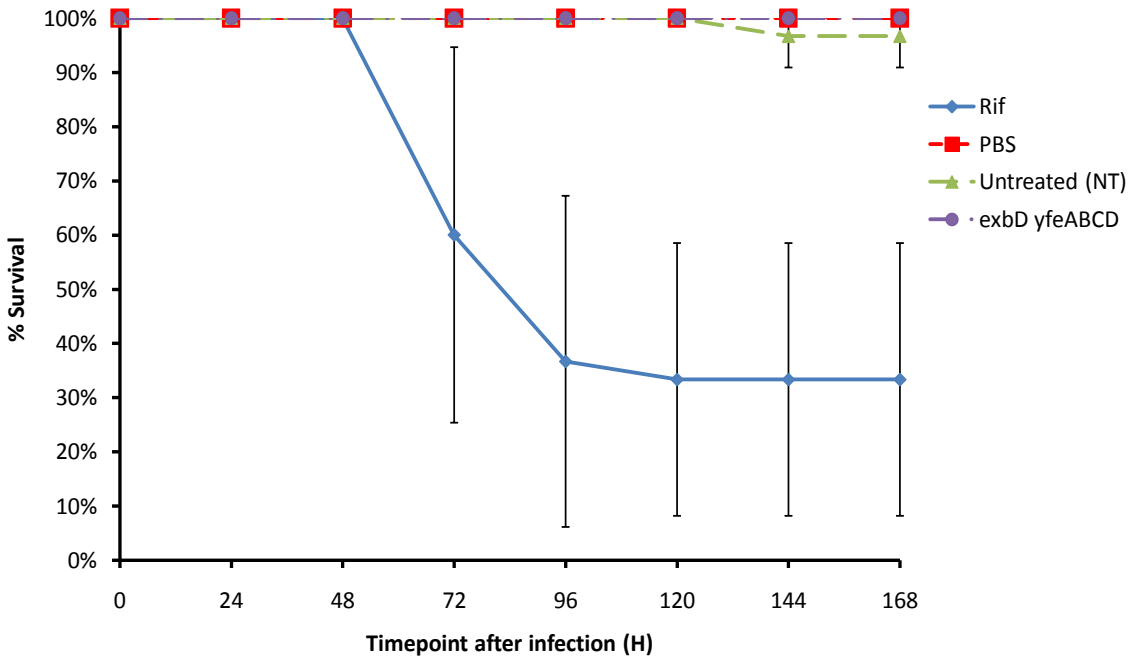

Figure 4.7 - Survival curve of *Manduca sexta* against the *exbD yfeABCD* double mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The 100% survival rate shows that *exbD yfeABCD* is unable to kill the caterpillar. The parent strain TT01 *Rif* is able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.7, the *exbD yfeABCD* double knock-out is unable to kill *M. sexta*. The parent strain *Rif* is able to kill approximately two-thirds of the insects. As shown previously in Figures 4.3 and 4.5, the single knock-out mutants of these genes are unable to kill any insects so the loss in pathogenicity of this *exbD yfeABCD* double knock-out mutant is due in equal measure to loss of both genes. This result is consistent with the results of Figs. 4.3 and 4.5.

The next mutant to be injected was a *feoAB yfeABCD* double knock-out. *feoAB* is an integral membrane protein that has a role in iron (II) up-take. *yfeABCD* has a role in transporting iron (II) and Manganese into the cell.

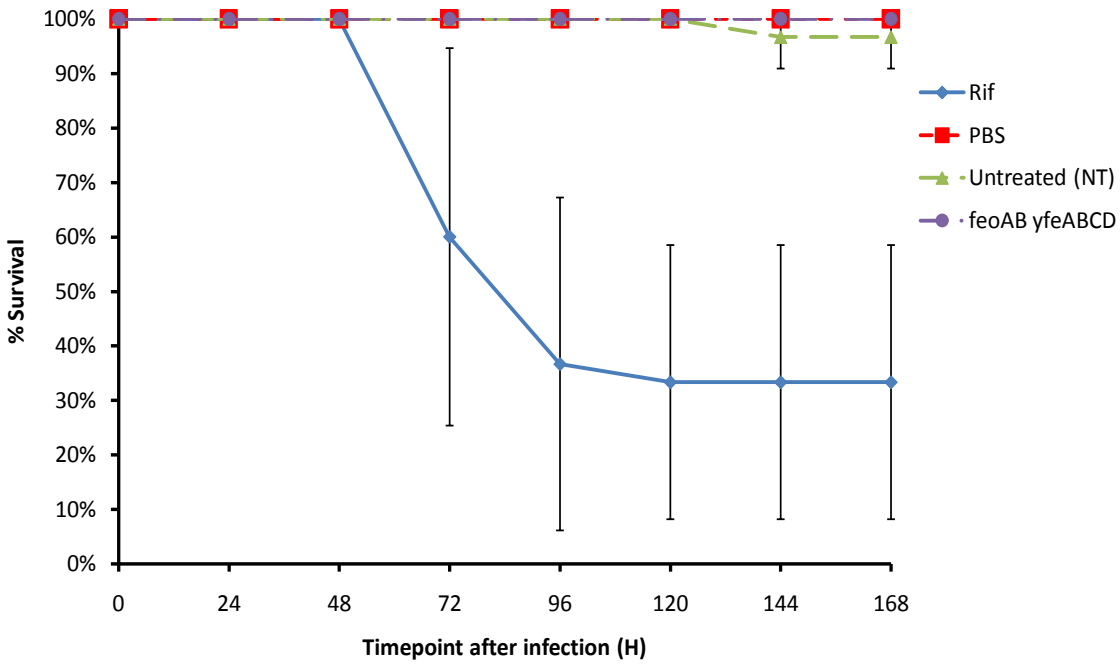

Figure 4.8 - Survival curve of *Manduca sexta* against the *feoAB yfeABCD* double mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The 100% survival rate shows that *feoAB* y*feABCD* is unable to kill the caterpillar. The parent strain TT01 *Rif* is able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.8, the *feoAB yfeABCD* double knock-out is unable to kill *M. sexta*. The parent strain *Rif* is able to kill approximately two-thirds of the insects. This loss in pathogenicity is probably due to the *yfeABCD* knock-out rather than the loss of both genes because as shown previously in Figure 4.5, the *yfeABCD* single knock-out is unable to kill any insects. This indicates that *yfeABCD* has a strong role in the pathogenicity of *P. luminescens* strain TT01. This result is an independent confirmation of the result seen in Fig. 4.5.

The next mutant to be injected was an *exbD feoAB yfeABCD* triple knock-out. *exbD* is a membrane bound protein which forms a complex with *TonB* and *exbB* and is essential for ferric ion up-take in bacteria. *feoAB* is an integral membrane protein that has a role in iron (II) up-take. *yfeABCD* has a role in transporting iron (II) and Manganese into the cell

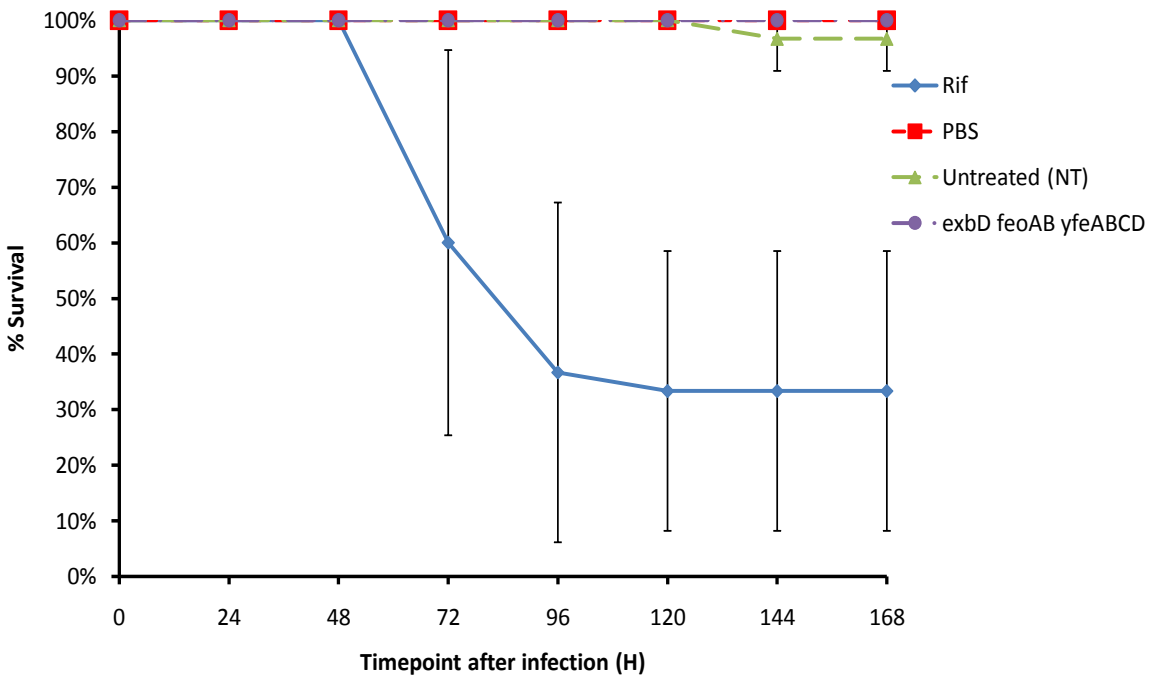

Figure 4.9 - Survival curve of *Manduca sexta* against the *exbD feoAB yfeABCD* triple mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The 100% survival rate shows that *exbD feoAB yfeABCD* is unable to kill the caterpillar. The parent strain TT01 *Rif* is able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.9, the *exbD feoAB yfeABCD* triple knock-out is unable to kill *M. sexta*. The parent strain *Rif* is able to kill approximately two-thirds of the insects. The loss of pathogenicity in this triple mutant is probably due in equal measure to the loss of *exbD* and *yfeABCD* genes. Consistent with the results shown previously in Figure 4.3 and 4.5, the single knock-outs of these genes were unable to kill any insects, while the single knock-out of *feoAB* was unaffected in its ability to kill (Figure 4.4).

The next mutant to be injected was an *ftnA* knock-out. This is a non-heme Ferritin protein responsible for the storage of iron.

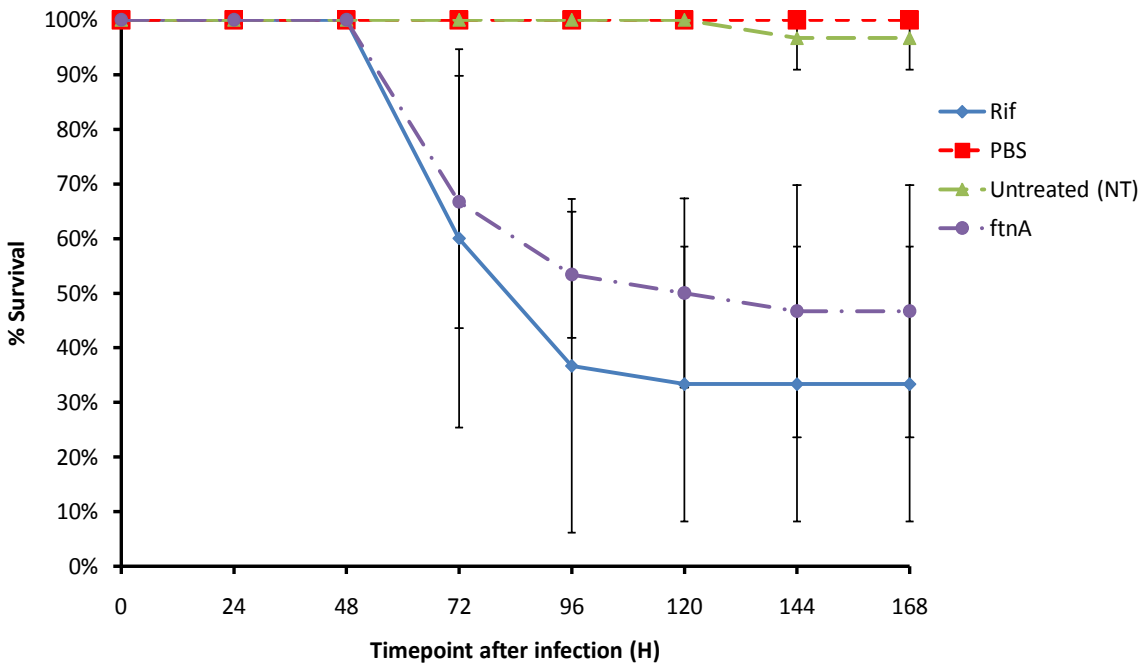

Figure 4.10 - Survival curve of *Manduca sexta* against the *ftnA* mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. *ftnA* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.10, the *ftnA* knock-out is able to kill *M. sexta* with similar efficacy to the parent strain *Rif*, which is able to kill approximately two-thirds of the insects. This indicates that *ftnA* has no significant role in pathogenicity of *P. luminescens* strain TT01.

The next mutant to be injected was a *ppxAB* knock-out. This is a Photopexin protein and is similar to iron storage proteins found in liver.

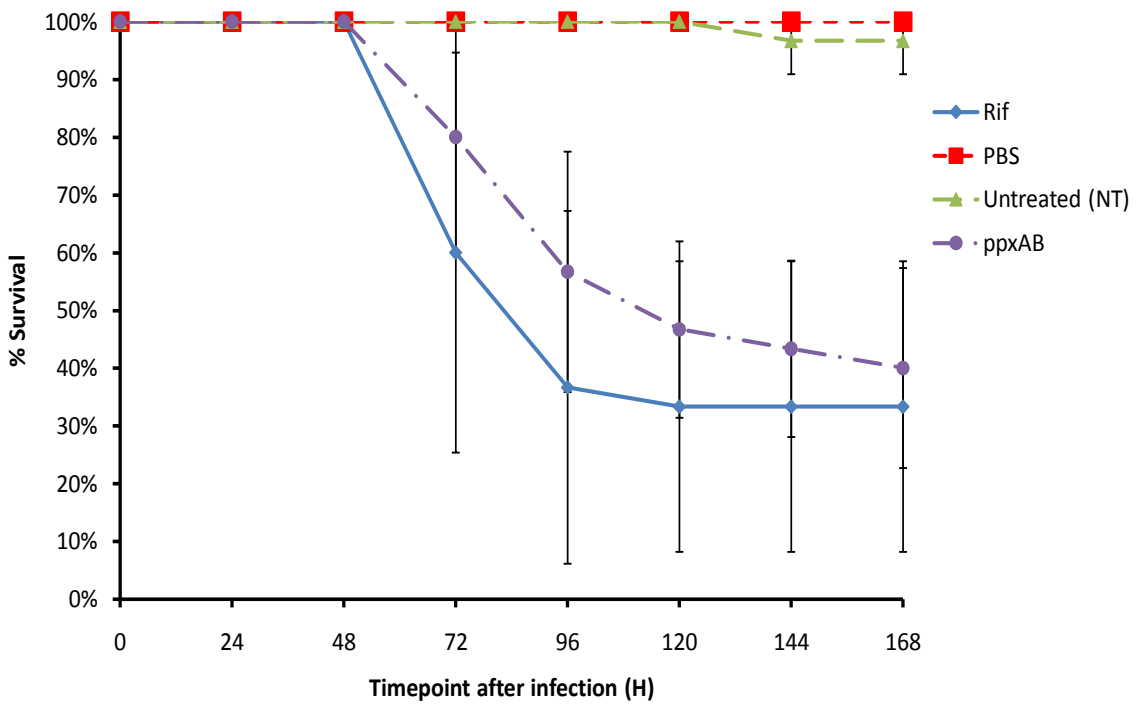

Figure 4.11 - Survival curve of *Manduca sexta* against the *ppxAB* mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. *ppxAB* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.11, the *ppxAB* knock-out is able to kill *M. sexta* with similar efficacy to the parent strain *Rif*, which is able to kill two-thirds of the insects. This indicates that *ppxAB* has no significant role in pathogenicity of *P. luminescens* strain TT01.

The next mutant to be injected was a *plu3613* knock-out. This protein shows similarly to a putative heme-binding protein of *Yesinia pestis*.

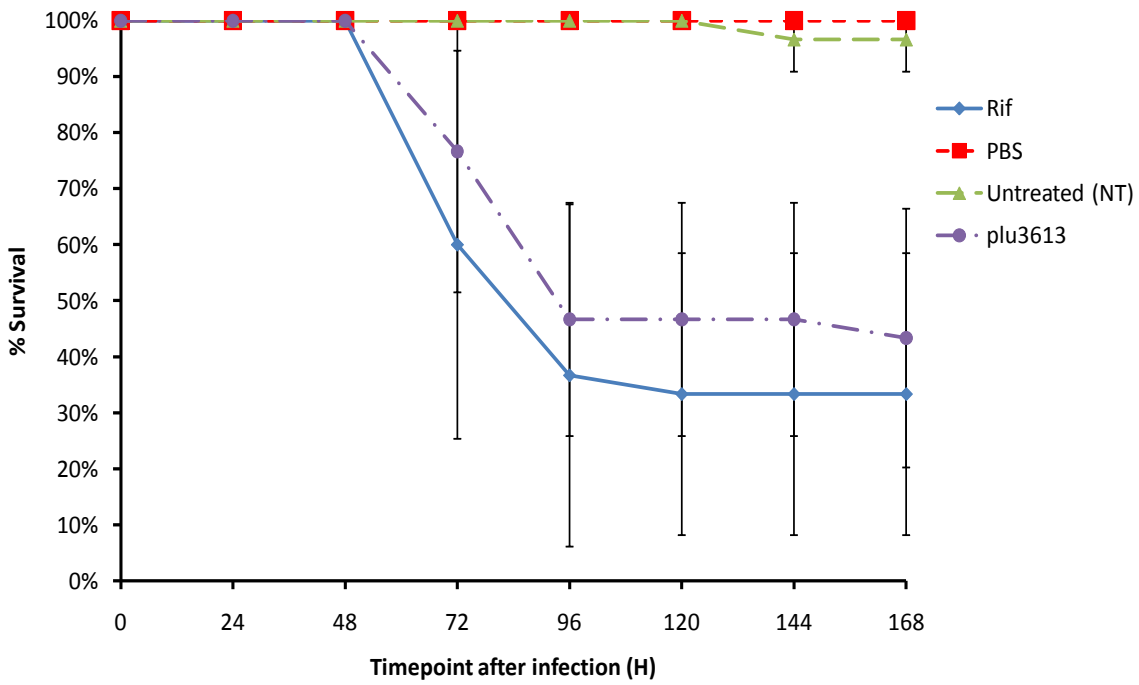

Figure 4.12 - Survival curve of *Manduca sexta* against the *plu3613* mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. *plu3613* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.12, the *plu3613* knock-out is able to kill *M. sexta* with a similar efficacy to the parent strain *Rif*, which is able to kill approximately two-thirds of the insects. This indicates that *plu3613* has no significant role in the pathogenicity of *P. luminescens* strain TT01.

The next mutant to be injected was a *plu4231* knock-out. This is a putative Photopexin protein and is similar to iron storage proteins found in liver.

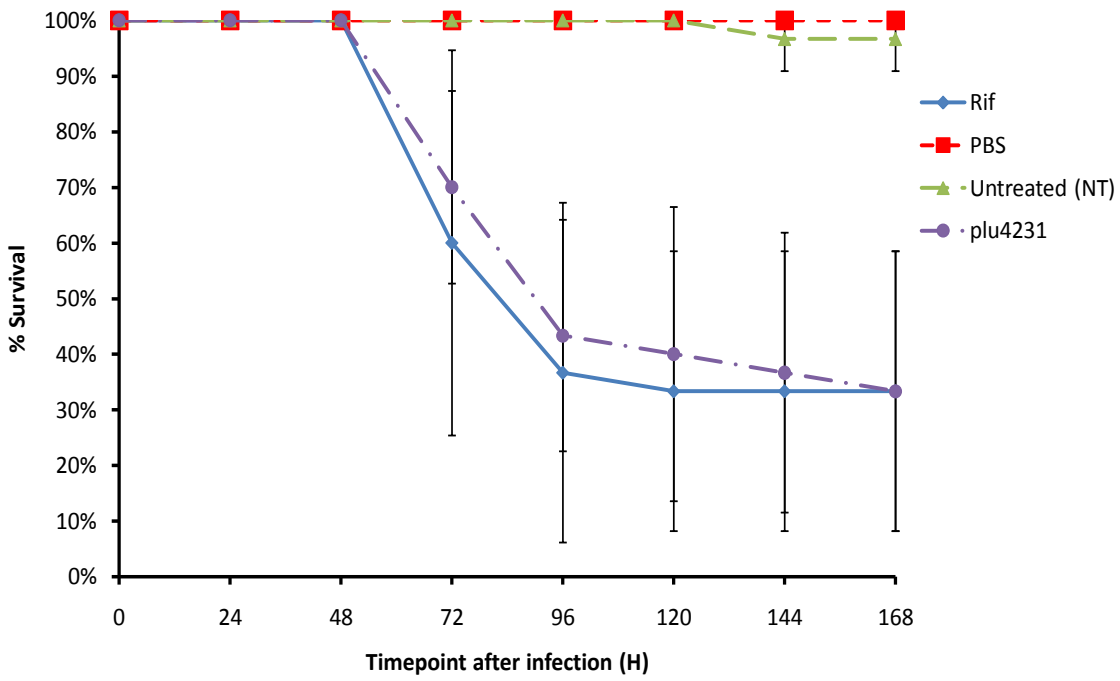

Figure 4.13 - Survival curve of *Manduca sexta* against the *plu4231* mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. *plu4231* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.13, the *plu4231* knock-out is able to kill *M. sexta* with similar efficacy to the parent strain *Rif*, which is able to kill two-thirds of the insects. This indicates that *plu4231* has no significant role in pathogenicity of *P. luminescens* strain TT01.

The final mutant to be injected was a *ppxAB plu4231* double knock-out. Both are Photopexin proteins and are similar to iron storage proteins found in liver.

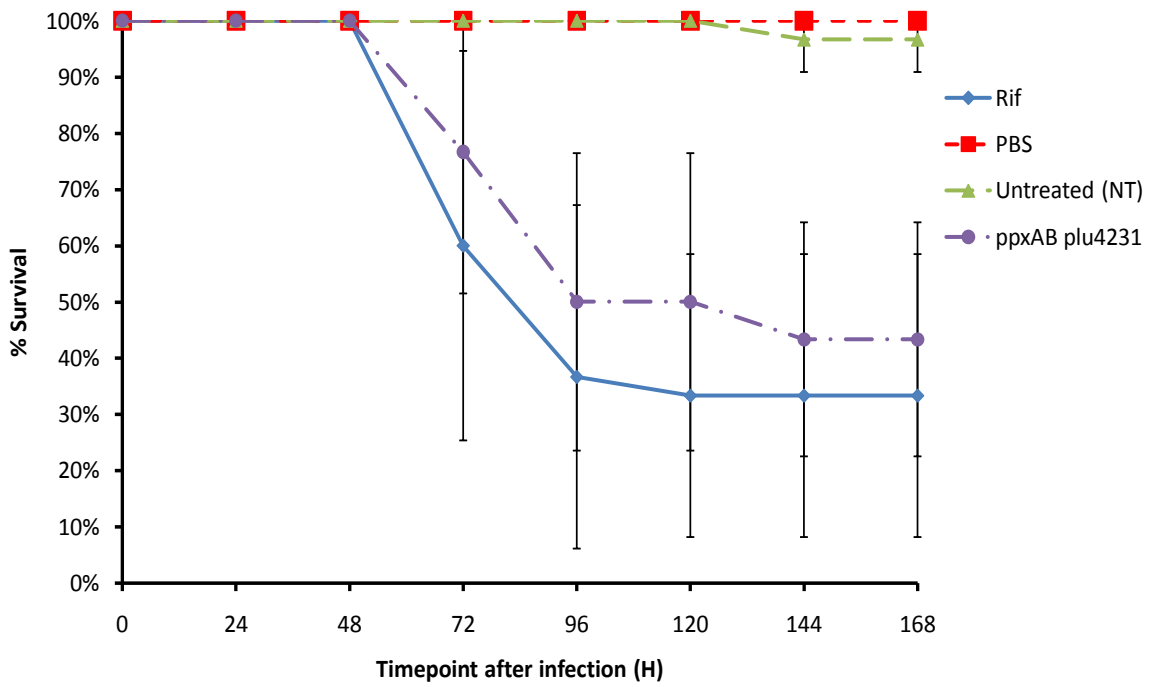

Figure 4.14 - Survival curve of *Manduca sexta* against the *ppxAB plu4231* double mutant of *Photorhabdus luminescens* strain TT01 over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. *ppxAB plu4231* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown by Figure 4.14, the *ppxAB plu4231* knock-out is able to kill *M. sexta* with similar efficacy to the parent strain *Rif*, which is able to kill two-thirds of the insects. This indicates that *ppxAB* or *plu4231* has no significant role in pathogenicity of *P. luminescens* strain TT01.

Figure 4.15 is a summary of the final survival percentages against the knock-out mutants.

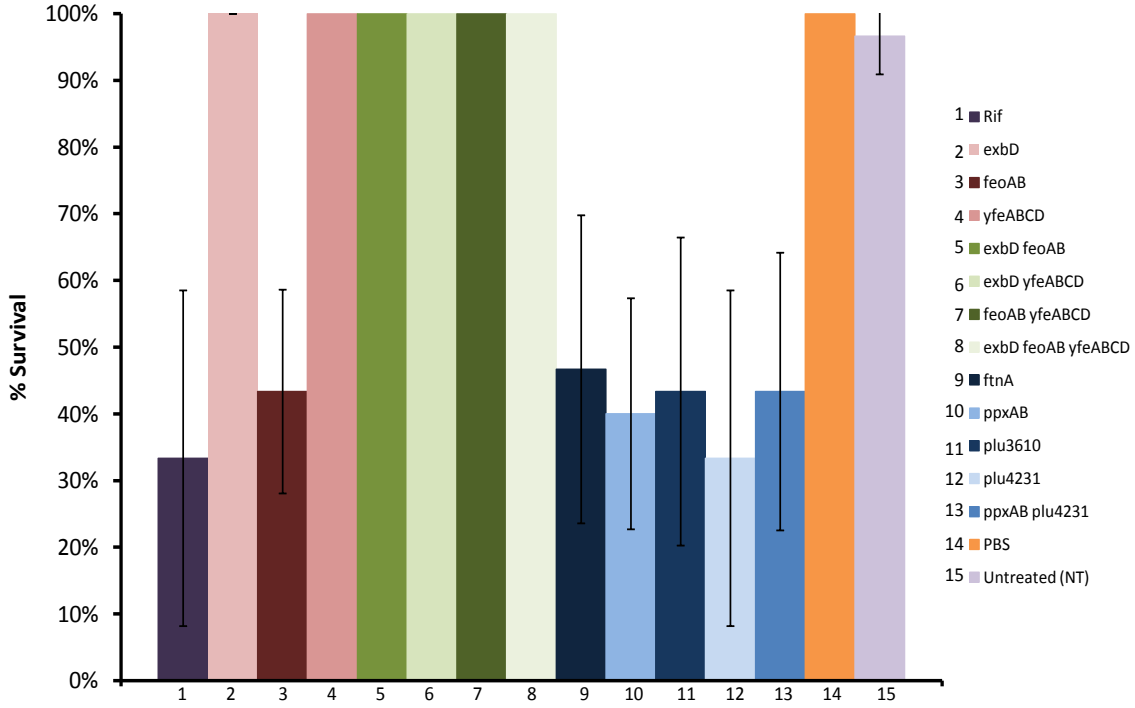

Figure 4.15 – Overview of the survival percentage of *Manduca sexta* against a number of *Photorhabdus luminescens* strain TT01 mutants. Columns represent mean values ± standard deviation (n=3 repeats) at 168 hours after injection. Ten insects were used for each treatment in each repeat experiment. The mutants; *exbD*, *yfeABCD*, double mutants; *exbD feoAB*, *exbD yfeABCD*, *feoAB yfeABCD* and the triple mutant; *exbD feoAB yfeABCD* are unable to kill *M. sexta* as shown by the 100% survival rate. The mutants; *feoAB*, *ftnA*, *ppxAB*, *plu3610*, *plu4231* and the double mutant; *ppxAB plu4231* killed the insects with similar efficacy to the parent strain TT01 *Rif* which was able to kill approximately two-thirds of the caterpillars. The controls (PBS and NT) show that the injection procedure without bacteria is harmless.

As shown in Figure 4.15, any knock-out in *exbD* or *yfeABCD* results in a loss of pathogenicity for *P. luminescens* strain TT01. This indicates that these two genes have strong roles in the pathogenicity of *P. luminescens* strain TT01. Every other gene investigated kills insects with a similar efficacy to the parent strain *Rif*, which was able to kill approximately two-thirds of the insects. This indicates that all these genes have no significant role in pathogenicity of *P. luminescens* strain TT01.

Iron and Manganese rescue of *Photorhabdus luminescens* strain TT01 knock-out mutants

To investigate if the loss of pathogenicity of the *exbD* and *yfeABCD* single knock-out mutants could be reversed by the introduction of excess Iron or Manganese, *M. sexta* was injected first with Iron or Manganese or a combination of both then the insect was injected with a *P. luminescens* strain TT01 mutant. Insects were checked for mortality once every 24 hours until 168 hours (7 days) after the initial injection.

First, *M. sexta* was injected with 5mM of Iron (III) chloride before injection with a *P. luminescens* strain TT01 mutant.

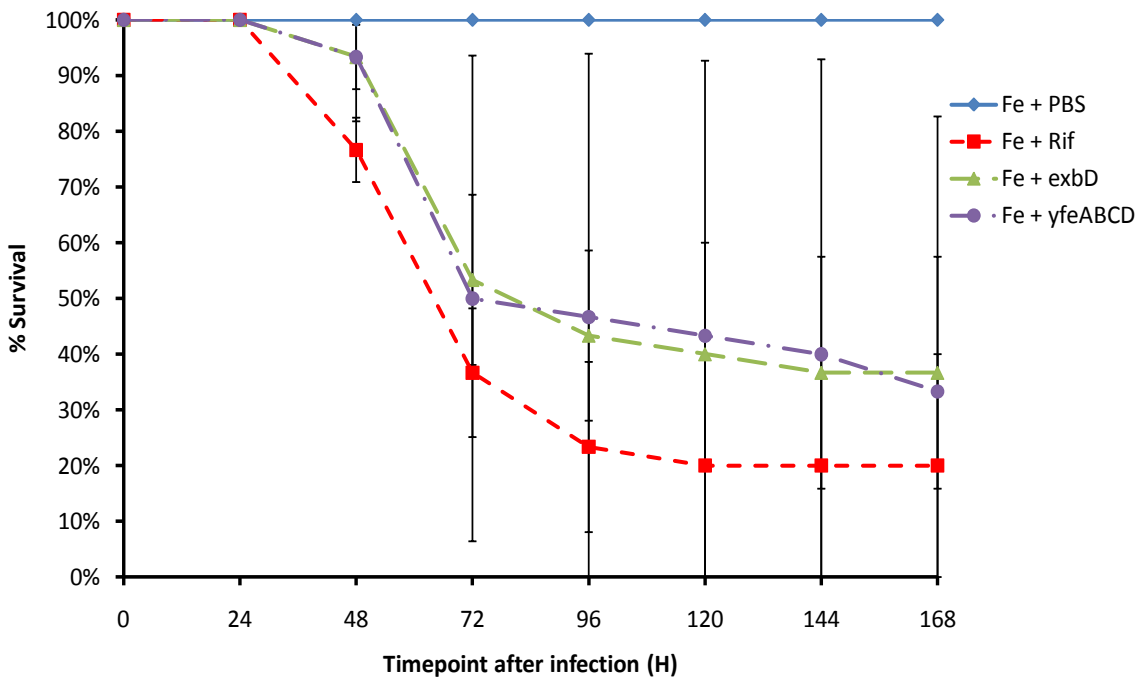

Figure 4.16 – Survival curve of iron treated *Manduca sexta* against *Photorhabdus luminescens* TT01 mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The previously ineffective mutants *exbD* and *yfeABCD* are now able to kill approximately two thirds of the insects while the *Rif* parent mutant strain is able to kill approximately three-quarters of the insects. The PBS control shows that the injection procedure with excess iron and without bacteria is harmless.

As shown by Figure 4.16, the *exbD* and *yfeABCD* knock-out mutants are able to kill approximately two-thirds of the insects when there is an excess of Iron. The parent strain *Rif* is able to kill approximately three-quarters of the insects. The excess Iron is able to rescue the loss of pathogenicity that both of the knock-out mutants suffer from.

Next, *M. sexta* was injected with 5mM of Manganese (II) chloride before injection with a *P. luminescens* strain TT01 mutant.

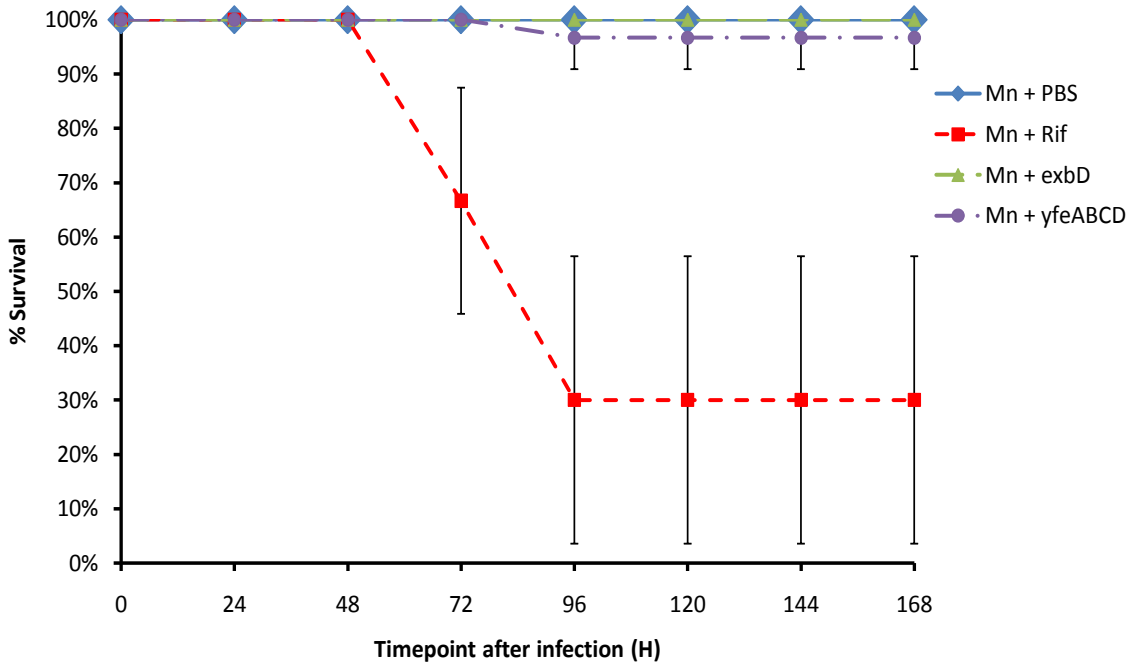

Figure 4.17 – Survival curve of manganese treated *Manduca sexta* against *Photorhabdus luminescens* TT01 mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. Both mutants *exbD* and *yfeABCD* remain ineffective whereas the *Rif* parent mutant strain is able to kill approximately two-thirds of the insects. The PBS control shows that the injection procedure with excess manganese and without bacteria is harmless.

As shown by Figure 4.17, the *exbD* and *yfeABCD* were unable to kill any insects in the presence of excess Manganese. The parent strain *Rif* is able to kill approximately two-thirds of the insects. This concentration of excess Manganese is unable to rescue the knock-out mutants from a loss of pathogenicity.

Finally, *M. sexta* was injected with 5mM of Iron (III) chloride and Manganese (II) chloride solution before injection with a *P. luminescens* strain TT01 mutant.

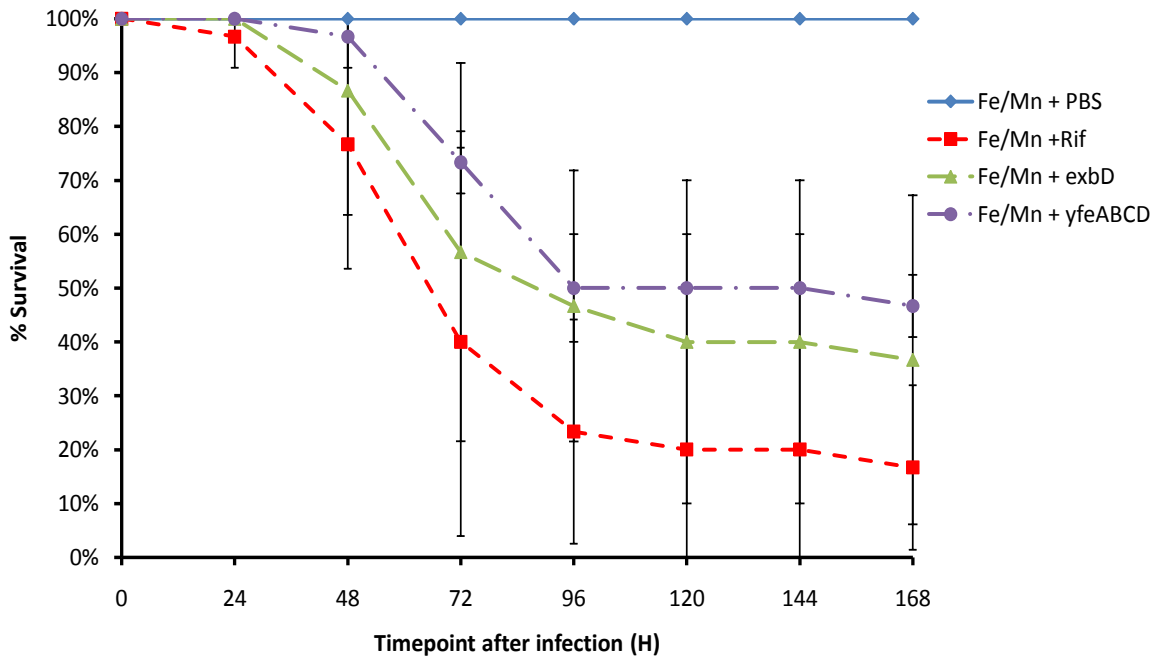

Figure 4.18 – Survival curve of iron and manganese treated *Manduca sexta* against *Photorhabdus luminescens* TT01 mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. The previously ineffective mutants *exbD* and *yfeABCD* are now to able kill approximately two thirds of the insects while the *Rif* parent mutant strain is able to kill approximately three-quarters of the insects. The PBS control shows that the injection procedure with excess iron plus manganese and without bacteria is harmless.

As shown by Figure 4.18, the *exbD* and *yfeABCD* knock-out mutants are able to kill approximately two-thirds of the insects. The parent strain *Rif* is able to kill approximately three-quarters of the insects. This concentration of Iron and Manganese is able to rescue the knock-out mutant's loss of pathogenicity, although this is probably due to the concentration of excess Iron rather than Manganese, because as shown previously, this concentration of Manganese is unable to rescue the knock-out mutant's loss of pathogenicity. The results of this experiment are an independent confirmation of the result previously reported in Fig. 4.16.

Figure 4.19 is a summary of the final survival percentage of attempted Iron and Manganese rescue of *P. luminescens* strain TT01 mutants.

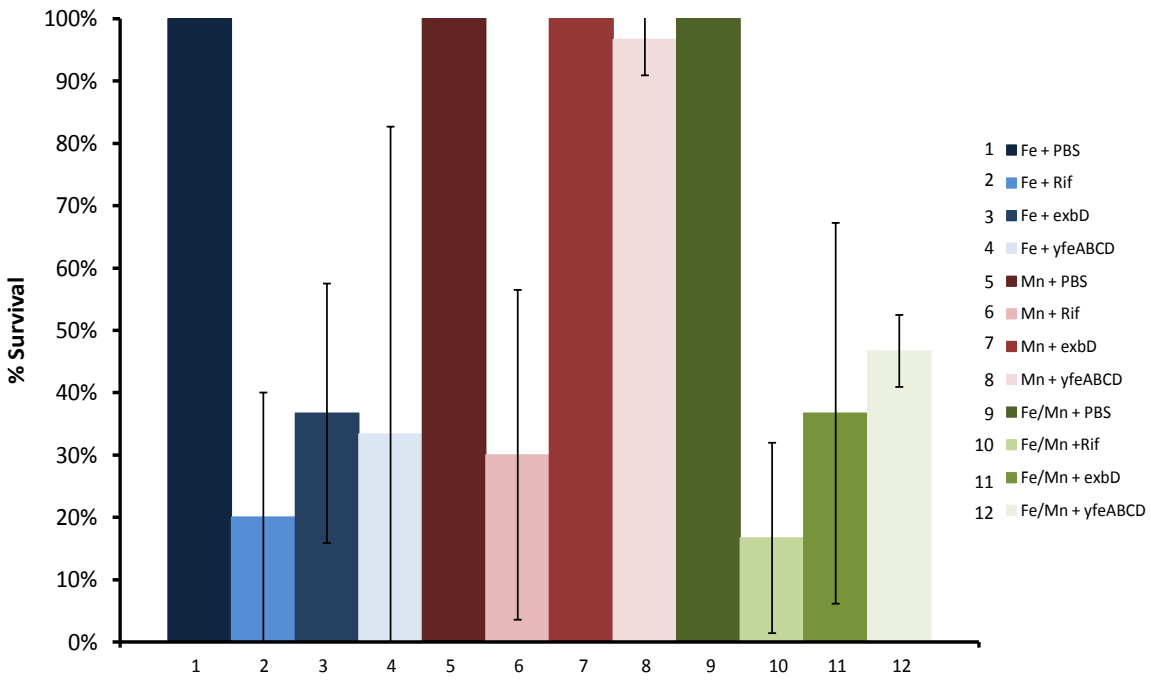

Figure 4.19 - Overview of the survival percentage of *Manduca sexta* against *Photorhabdus luminescens* strain TT01 mutants *exbD* and *yfeABCD* after having been injected with excess iron (Fe), manganese (Mn) or both(Fe/Mn). Columns represent mean values ± standard deviation (n=3 repeats) at 168 hours after injection. Ten insects were used for each treatment in each repeat experiment. Treating the insects with just iron restored pathogenicity to both mutants resulting in them killing approximately two-thirds of the insects. Treating the insects with just manganese failed to restore pathogenicity to both mutants as shown by the ~100% survival rate. Treating the insects with both iron and manganese restores pathogenicity to both mutants resulting in them killing approximately two-thirds of the insects. The *Rif* parent mutant strain is able to kill approximately three-quarters of the insects in all cases. The PBS control shows that the injection procedure with either iron or manganese or both and without bacteria is harmless.

As shown in Figure 4.19, the *exbD* and *yfeABCD* knock-out mutants are able to kill approximately two-thirds of the insects when co-injected with excess Iron. However when injected with excess Manganese at a concentration of 5mM, the *exbD* and *yfeABCD* knock-out mutants are unable to kill any insects. Injecting both Iron and Manganese results in the *exbD* and *yfeABCD* knock-out mutants being able to kill approximately two-thirds of the insects. It's clear that injecting 5mM of Iron into *M. sexta* is able to rescue the loss of pathogenicity suffered by the *exbD* and *yfeABCD*, while the same concentration of Manganese has no effect on these mutants. Injecting both metals together does again rescue the loss of pathogenicity, but this is probably due to the presence of excess Iron rather than Manganese. This also indicates that *exbD* and *yfeABCD* ability to up-take and transport Iron is essential to the pathogenicity of *P. luminescens* strain TT01.

Injecting with double-stranded RNA does not increase susceptibility to *Photorhabdus luminescens* strain TT01

To investigate if knocking down the transcription of Transferrin and Ferritin would increase the susceptibility of *M. sexta* to the *exbD* and *yfeABCD* knock-out mutants, *M. sexta* was injected with dsRNA for either Transferrin or Ferritin, and then 24 hours later, the insects were injected with *P. luminescens* strain TT01. Insects were checked for mortality once every 24 hours until 168 hours (7 days) after the second injection.

First, *M. sexta* was injected with water before injection with *P. luminescens* strain TT01.

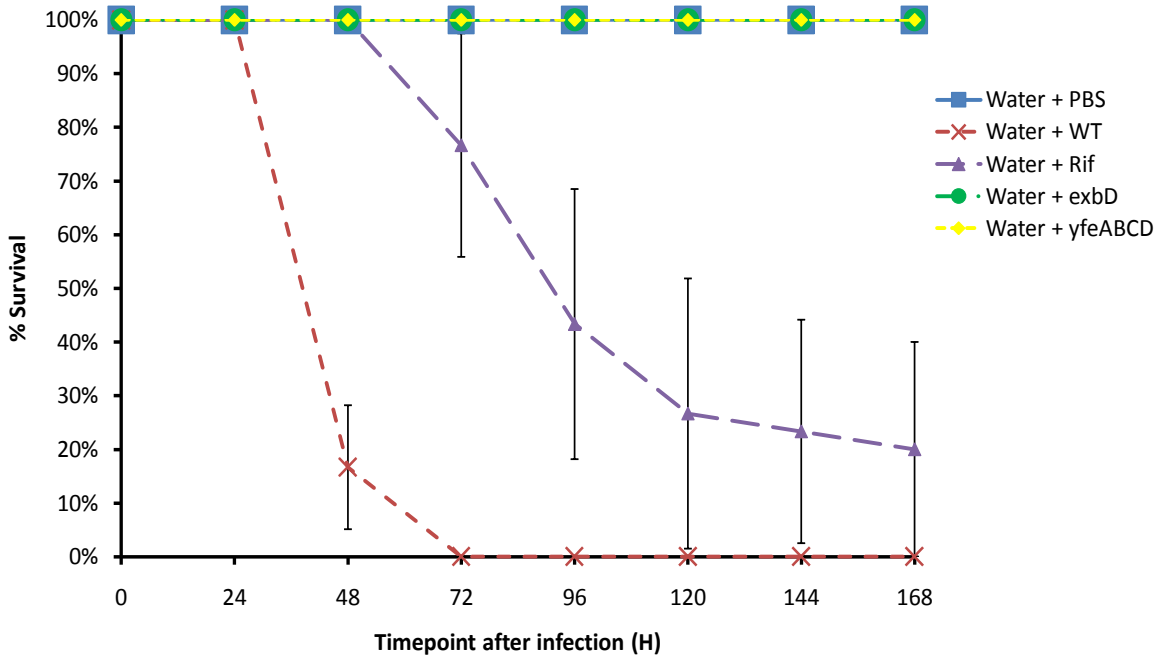

Figure 4.20 – Survival curve of endotoxin-free water treated *Manduca sexta* against *Photorhabdus luminescens* strain TT01 and three mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. Injecting the caterpillars with the wild-type (WT) strain killed all of the insects within 72 hours (H) of exposure. Similarly, injecting the *Rif* parent mutant strain resulted in three-quarters of the insects being killed. The other mutants; *exbD* and *yfeABCD* failed to kill any insects. The PBS control shows that the injection procedure without bacteria is harmless.

As shown by Figure 4.20, the wild-type strain is able to kill all the insects by 72 hours after the second injection. The *Rif* mutant is able to kill approximately three-quarters of the insects. As previously noted, the *Rif* mutant is significantly less virulent than the parent wild type TT01 strain. As shown previously, the *exbD* and *yfeABCD* are unable to kill any insects. This shows that water does not increase the susceptibility of *M. sexta* to *P. luminescens* strain TT01.

Next, *M. sexta* was injected with a control dsRNA before injection with *P. luminescens* strain TT01.

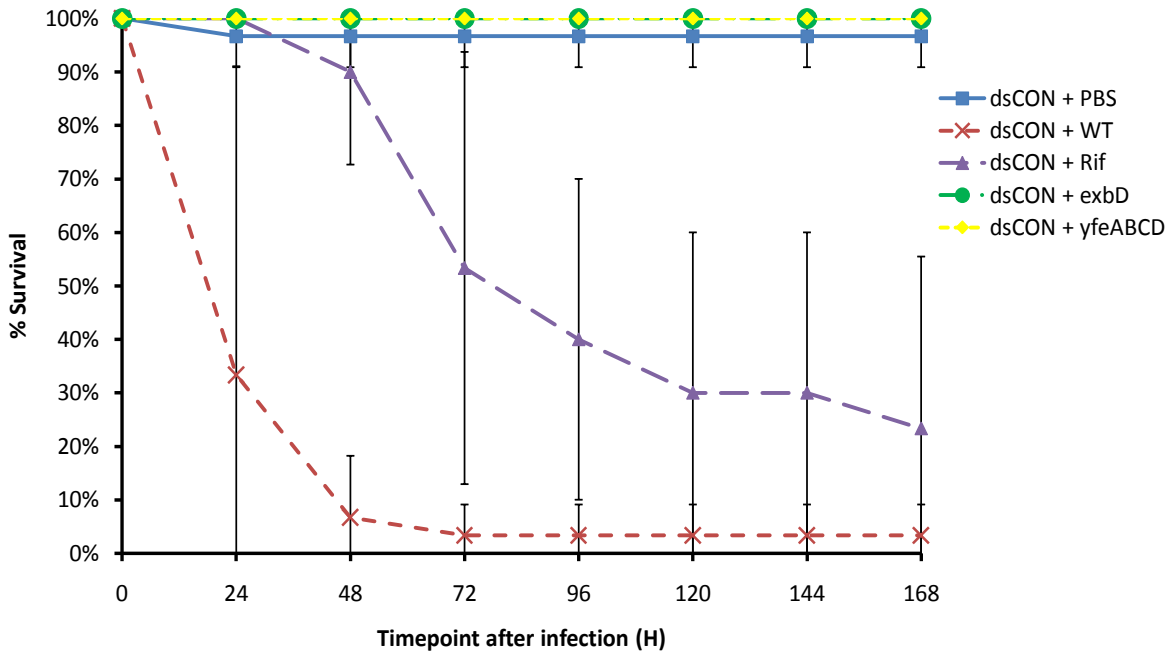

Figure 4.21 – Survival curve of control dsRNA (dsCON) treated *Manduca sexta* against *Photorhabdus luminescens* strain TT01 and three mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. Injecting the caterpillars with the wild-type (WT) strain killed most of the insects within 72 hours (H) of exposure. Similarly, injecting the *Rif* parent mutant strain resulted in three-quarters of the insects being killed. The other mutants; *exbD* and *yfeABCD* failed to kill any insects. The PBS control shows that the injection procedure without bacteria is harmless.

As shown by Figure 4.21, the wild-type strain is able to kill most of the insects by 72 hours after the second injection. The *Rif* mutant is able to kill approximately three-quarters of the insects. As shown previously, the *exbD* and *yfeABCD* are unable to kill any insects. This shows that the control dsRNA does not increase the susceptibility of *M. sexta* to *P. luminescens* strain TT01.

Next, *M. sexta* was injected with dsRNA for Transferrin before injection with *P. luminescens* strain TT01.

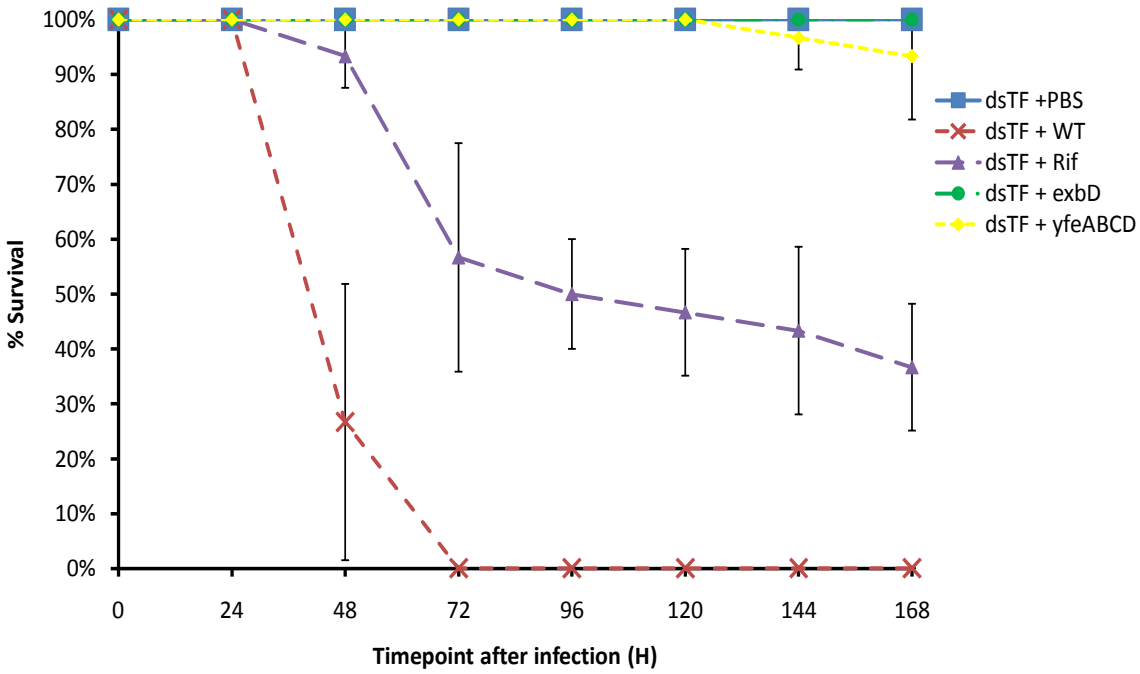

Figure 4.22 – Survival curve of dsRNA for Transferrin (dsTF) treated *Manduca sexta* against *Photorhabdus luminescens* strain TT01 and three mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. Injecting the caterpillars with the wild-type (WT) strain killed all of the insects within 72 hours (H) of exposure. Similarly, injecting the *Rif* parent mutant strain resulted in two-thirds of the insects being killed. The other mutants; *exbD* and *yfeABCD* failed to kill any insects. The PBS control shows that the injection procedure without bacteria is harmless.

As shown by Figure 4.22, the wild-type strain is able to kill all the insects by 72 hours after the second injection. The *Rif* mutant is able to kill approximately two-thirds of the insects. The *exbD* and *yfeABCD* remain unable to kill any insects. This shows that the injection of dsRNA for Transferrin does not increase the susceptibility of *M. sexta* to *P. luminescens* strain TT01.

Finally, *M. sexta* was injected with dsRNA for Ferritin before injection with *P. luminescens* strain TT01.

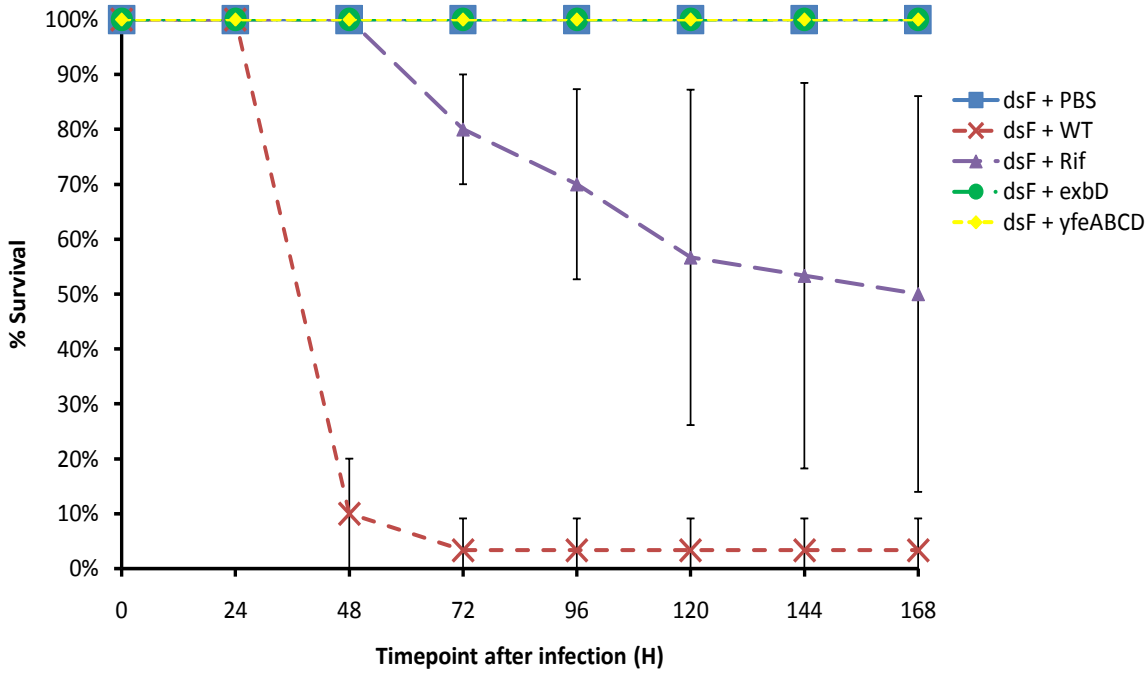

Figure 4.23 – Survival curve of Ferritin dsRNA (dsF) treated *Manduca sexta* against *Photorhabdus luminescens* strain TT01 and three mutants over a time period of 168 hours (H). Points show mean values ± standard deviation (n=3 repeats). Ten insects were used for each treatment in each repeat experiment. Injecting the caterpillars with the wild-type (WT) strain killed most of the insects within 72 hours (H) of exposure. Similarly, injecting the *Rif* parent mutant strain resulted in half of the insects being killed. The other mutants; *exbD* and *yfeABCD* failed to kill any insects. The PBS control shows that the injection procedure without bacteria is harmless.

As shown by Figure 4.23, the wild-type strain is able to kill most of the insects by 72 hours after the second injection. The *Rif* mutant is able to kill approximately half of the insects. The *exbD* and *yfeABCD* remain unable to kill any insects. This shows that the injection of dsRNA for Ferritin does not increase the susceptibility of *M. sexta* to *P. luminescens* strain TT01.

Figure 4.24 is a summary of the final survival percentage of dsRNA treated *M. sexta* against *P. luminescens* strain TT01.

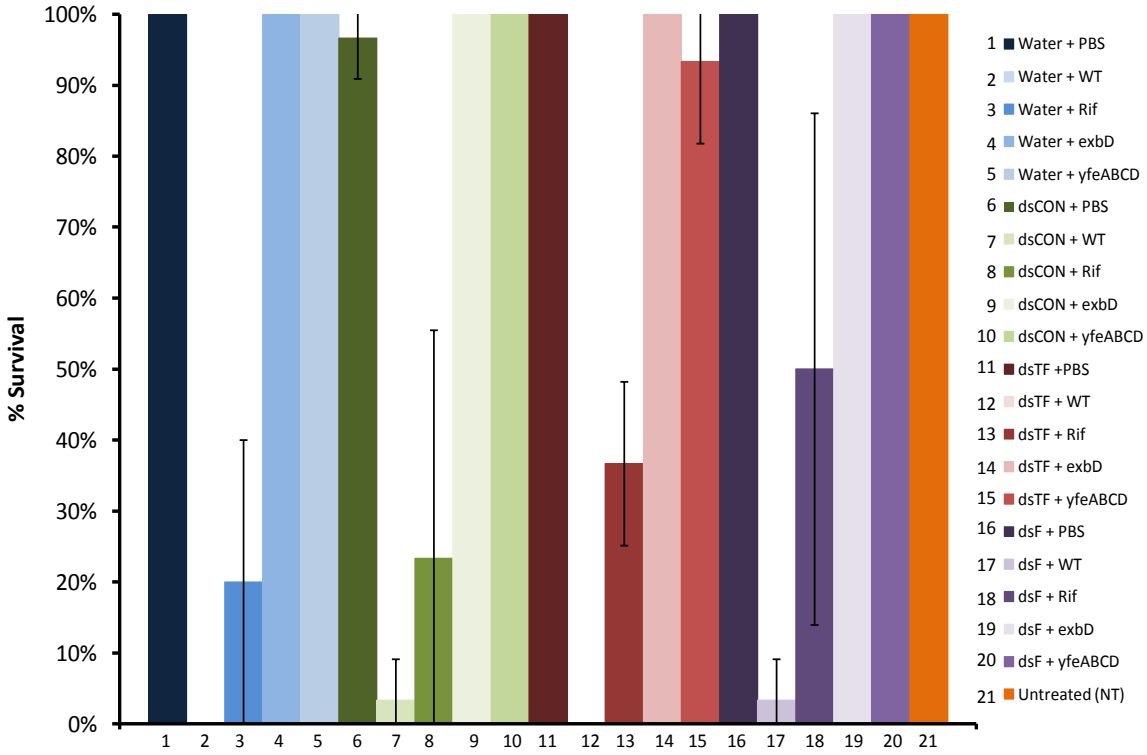

Figure 4.24 – Overview of the survival percentage of *Manduca sexta* against *Photorhabdus luminescens* strain TT01 and three mutant strains after being injected with dsRNA. Columns represent mean values ± standard deviation (n=3 repeats) at 168 hours after injection. Ten insects were used for each treatment in each repeat experiment. Injecting *M. sexta* with dsRNA for both Transferrin (dsTF) and Ferritin (dsF) had no effect on their susceptibility towards the mutants; *exbD* and *yfeABCD* as shown by the 100% survival rates. Injecting dsTF or dsF also did not increase the insect's susceptibility towards the wild-type (WT) or to the *Rif* mutant either with no difference between the controls; injected with either endotoxin-free water or a control dsRNA (dsCON), and the experimental; those injected with dsTF or dsF. The PBS control shows that the injection procedure without bacteria is harmless.

As shown in Figure 4.24, the injection of dsRNA for both Transferrin and Ferritin had no effect on the susceptibility of *M. sexta* to *P. luminescens* strain TT01. Injecting dsRNA had no effect on the time taken by the wild-type to kill most of the insects either, with similar curves exhibited by all treatments.

Transferrin mRNA is not reduced following dsRNA treatment

To determine if Transferrin mRNA was reduced following an injection with dsRNA for Transferrin, RNA was isolated from fat body of *M. sexta* injected with dsRNA for Transferrin, left for 24 hours, then injected with *E. coli*, and then left for a further 24 hours. RT-PCR was used to detect Transferrin mRNA.

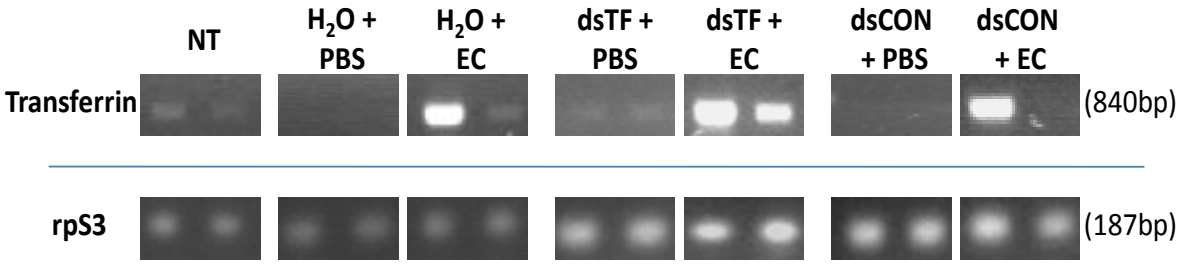

Figure 4.25 – Expression of Transferrin mRNA in *Manduca sexta* after injection of dsRNA for Transferrin (dsTF). The panels show RT-PCR bands from pairs of similarly-treated insects. There does not appear to be a knockdown in expression of Transferrin as the intensity of the bands between those treated with PBS and the untreated controls (NT) seem similar. Also band intensity between those challenged with EC does not differ by very much. Any differences that do arise are probably due to other factors unrelated to the dsRNA treatment. This experiment was repeated with a different set of insects and the results found to be the same. rpS3 mRNA levels are included as a loading control.

As shown in Figure 4.25, Transferrin mRNA was not reduced by treatment with dsRNA for Transferrin. Treatment with a control dsRNA or endotoxin-free water also did not reduce Transferrin mRNA. All insects injected with *E. coli* show up-regulation of transcription of Transferrin mRNA. This indicates that an RNAi knockdown of Transferrin at the mRNA level was not achieved in this experiment.

Knockdown of Ferritin mRNA following dsRNA treatment

To determine if Ferritin mRNA was reduced following an injection with dsRNA for Ferritin, RNA was isolated from fat body of *M. sexta* injected with dsRNA for Ferritin, left for 24 hours, then injected with *E. coli*, and then left for a further 24 hours. RT-PCR was used to detect Ferritin mRNA.

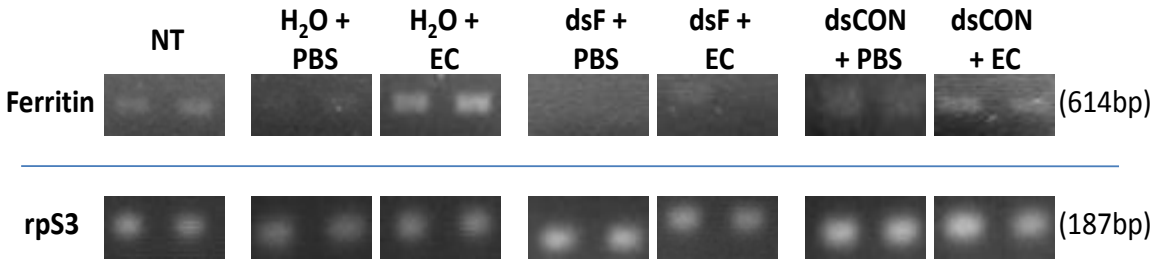

Figure 4.26 – Expression of Ferritin mRNA in *Manduca sexta* after injection of dsRNA for Ferritin (dsF). The panels show RT-PCR bands from pairs of similarly-treated insects. There does appear to be a knockdown in expression of Ferritin as the intensity of the bands between the controls and the experimental seems to be reduced. There appears to be very faint bands in those treated with dsF whereas in all other treatments the intensity of the bands is slightly greater. rpS3 mRNA levels are included as a loading control.

As shown by Figure 4.26, Ferritin mRNA was reduced following an injection with dsRNA for Ferritin. Treatment with a control dsRNA or endotoxin-free water did not reduce Ferritin mRNA. As shown previously, injection with *E. coli* did not increase the level of Ferritin mRNA. This indicates there an RNAi knockdown of Ferritin was successfully achieved at the mRNA level.

Expression of Transferrin protein following dsRNA treatment

To determine if the expression of Transferrin is reduced following an injection with dsRNA for Transferrin, haemolymph was isolated from *M. sexta* injected with dsRNA for Transferrin, left for 24 hours, then injected with *E. coli*, and then left for a further 24 hours. A Western blot experiment was used to detect protein expression.

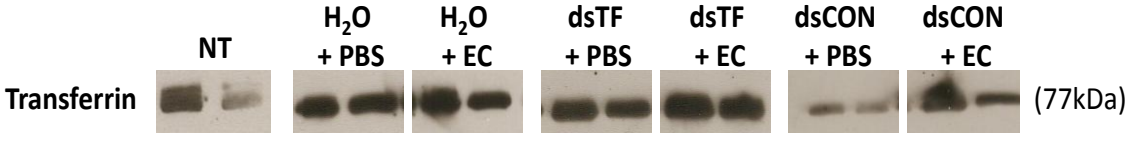

Figure 4.27 - Expression of Transferrin protein in *Manduca sexta* following injection of dsRNA for Transferrin (dsTF). Panels show bands from a Western blot. There appears to be no reduction in the protein level of Transferrin in those insects treated with dsTF compared to those insects not treated with dsTF in the other panels.

As shown by Figure 4.27, levels of Transferrin protein were not reduced following an injection with dsRNA. Treatment with a control dsRNA or endotoxin-free water

did not change the levels of Transferrin protein from constitutive levels. As expected from previous results, some of the insects injected with *E. coli* showed an increase in protein levels. One of the two H_2O control insects also given bacteria, and one of the two dsCON control insects also given bacteria, showed a clear upregulation of transferrin protein. Two of two insects given dsTF and then injected with *E. coli* showed clear upregulation. This is further evidence that an effective knockdown of Transferrin following dsRNA treatment was not achieved.

Expression of Ferritin protein following dsRNA treatment

To determine if the expression of Ferritin is reduced following an injection with dsRNA for Ferritin, haemolymph was isolated from *M. sexta* injected with dsRNA for Ferritin, left for 24 hours, then injected with *E. coli*, and then left for a further 24 hours. A Western blot experiment was used to detect protein expression.

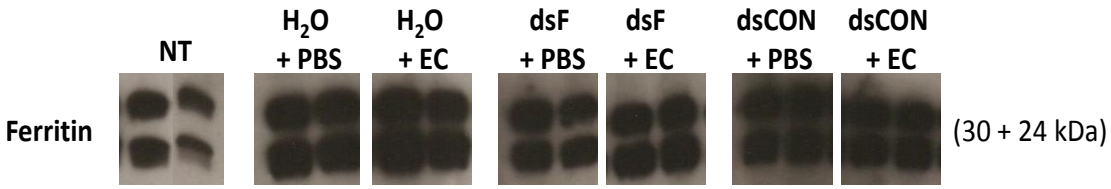

Figure 4.28 - Expression of Ferritin protein in *Manduca sexta* following injection of dsRNA for Ferritin (dsF). Panels show bands from a Western blot. There appears to be no reduction in the protein level of Ferritin in those insects treated with dsF compared to those insects not treated with dsF in the other panels.

As shown by Figure 4.28, levels of Ferritin protein were not reduced following an injection with dsRNA in this experiment. Treatment with a control dsRNA or endotoxin-free water did not change the levels of Ferritin protein from constitutive levels. Insects injected with *E. coli* show no increase in protein levels. This is indicates that there is no knockdown of Ferritin protein following dsRNA treatment.

Discussion

The main findings of this chapter were as follows:

- Injection of *E. coli* results in the increase of transferrin mRNA and protein levels, but ferritin mRNA and protein levels remain unchanged from controls.
- The knock-out of iron-uptake genes *exbD* and *yfeABCD* result in a loss of pathogenicity in the *Rif* mutant of *P. luminescens* strain TT01.

- The injection of ferric iron, but not manganese, reverses the loss of pathogenicity in the *P. luminescens* strain TT01 knock-out mutants, confirming that the loss of virulence experienced in the *exbD* and *yfeABCD* knock-out strains is due to interference with the requirement for iron.

The experiments used to test the prediction that the role in virulence of the *exbD* and *yfeABCD* genes were inconclusive, because the RNAi technique used in an attempt to knock down the expression of the insect host's iron sequestering mechanism was unsuccessful.

As discussed in the previous chapter, the recognition and the response to infection are very important for an organism's survival. Transferrin has been implicated to be part of the insect's immune defence by binding any free iron present within the body rather than for transport of iron (Law, 2002). Here, it was found that both transferrin mRNA and protein levels are up-regulated from constitutive levels in response to challenge from *E. coli* (Figure 4.1, Figure 4.2). This is consistent with previous findings in other insects, where infection has been shown to result in the up-regulation of transferrin.

This suggests that transferrin has some role in the immune response of *M. sexta*. Although it is unclear at exactly what time mRNA levels start to increase, protein levels are possibly up-regulated at around four hours, and show a big increase from 18 hours after infection. At 24 and 48 hours after infection, the levels of transferrin still appear to be increasing (Figure 4.2). The reason for this large and prolonged increase in transferrin levels is unclear; it is possible that the insect is still trying to maintain an iron-restrictive environment until all sign of the infection has gone, and that 48 hours is not enough time to rid the haemolymph of all the bacteria. Alternatively, it could be a developmental effect, although uninfected insects of the same age show no sign of transferrin up-regulation, so that this is unlikely. It is also possible that the clearing of the *E. coli* infection may release iron from bacterial stores. Such an increased availability of iron might have affected transferrin expression directly, and independently of the presence of bacterial elicitors. The *E. coli* that were injected had been previously grown in LB media where iron availability would have been unrestricted and therefore the bacteria could have built up a store of iron, which is released when the humoral and cellular defences of *M.*

sexta started to kill the bacteria. In contrast, no change in ferritin mRNA or protein levels was detected by RT-PCR or Western blot in response to *E. coli* infection. This suggests that unlike ferritin in *D. melanogaster*, ferritin in *M. sexta* has no role in the immune defence.

As described above, bacteria and in particular *P. luminescens* strain TT01 have many mechanisms by which to obtain iron from their current environment. To investigate some of these mechanisms, knock-out mutants of genes involved in the transport and storage of iron were injected into *M. sexta* and observed for any loss of pathogenicity compared to the parent rifamycin-resistant strain. Out of the 13 knock-out mutants tested, only those missing the *exbD* and *yfeABCD* genes were affected; single mutants *exbD, yfeABCD;* double mutants *exbD feoAB, exbD yfeABCD, feoAB yfeABCD;* and triple mutant *exbD feoAB yfeABCD* (Figures 4.3 – 4.14).

The *exbD* gene encodes ExbD protein, which forms part of the TonB complex. The TonB complex is used to provide energy for the transport of ferri-siderophores across the outer membrane of Gram-negative bacteria. Knock-out of the *exbD* gene results in a total loss of pathogenicity in *P. luminescens* strain TT01, indicating that it is essential for the pathogenesis of the bacterium. It also indicates that *P. luminescens* strain TT01 has a requirement for ferric iron at some point of the infection process, and/or simply needs it to grow.

The *yfeABCD* gene encodes proteins constituting a ferrous iron uptake mechanism which have homology to the same proteins first found in the plague bacterium *Yersinia pestis*. Similarly to the *exbD* knock-out, the loss of the *yfeABCD* results in a complete loss of pathogenicity in *P. luminescens* strain TT01, indicating that it is essential for the pathogenesis of the bacterium. This also suggests that *P. luminescens* strain TT01 has a requirement for ferrous iron at some point during pathogenesis. Interestingly, in a mouse model of bubonic plague *Y. pestis* was shown to require a Yersinabactin (siderophore-dependent) system in the early stages of infection, while its Yfe system was required for the later stages of infection. The present results suggest that a similar system may operate in the infection model of *P. luminescens* strain TT01.

Next, I attempted to rescue or reverse this loss of pathogenicity in *exbD* and *yfeABCD* knock-out mutants by injecting iron, manganese or a combination of both

along with the mutant. The injection of iron or the combination of iron and manganese resulted in the *exbD* and *yfeABCD* knock-out mutants regaining their pathogenicity (Figure 4.16, Figure 4.18). The injection of manganese however, did not reverse the loss of pathogenicity in either knock-out mutant (Figure 4.17).

These results suggest that iron is needed for the pathogenesis of *P. luminescens* strain TT01 against *M. sexta* and the loss of pathogenicity in the knock-out mutants is due to the inability of these bacteria to transport iron or iron-containing molecules into the cytosol. The results also suggest either that manganese is not required for the pathogenicity of *P. luminescens* strain TT01, or that the *yfeABCD* system is not essential for the uptake of manganese.

As transferrin expression evidently forms part of the immune response of *M. sexta* against infection, having been shown to be up-regulated in response to infection with *E. coli* (Figure 4.1), it was hypothesised that RNAi-mediated knock down of transferrin or ferritin might be able to reverse the loss of pathogenicity in the *exbD* and *yfeABCD* knock-out mutants. Injection of dsRNA for each particular gene resulted in no reversal in the loss of pathogenicity and no reduction in the time it took for a wild-type strain to kill most of the insects in contrast to knock down of PRRs (Figure 4.20 – 4.23). Further investigation revealed that there was no reduction of transferrin mRNA and protein levels and although there was reduction of ferritin mRNA levels, there was no reduction of ferritin protein levels after injection with the corresponding dsRNA. This result was surprising at the time, as in a number of previous studies performed in this laboratory, the same RNAi technique had been successful in knocking down a large number of immune related genes in *M. sexta*. Despite a number of attempts to determine the nature of the problem with the RNAi technique, it has not since been possible to obtain reliable RNAi gene silencing in *Manduca* (This unfortunate problem will be discussed further in the final chapter).

In conclusion, the work described in this Chapter has shown that *P. luminescens* strain TT01 requires iron as part of its pathogenesis against *M. sexta* and the two uptake mechanisms, and that *exbD* and *yfeABCD* are crucial to the bacterium's ability to obtain this valuable resource. The role of transferrin in the immune defence is still not clear however and it is apparent that further work needs to be done to elucidate this.

Chapter 5 – Investigating the cellular immune response of *Manduca sexta* using fluorescent-activated cell sorting

Introduction

The cellular immune defence of insects refers to the haemocyte-mediated responses of phagocytosis, nodulation and encapsulation. Although it should be noted that haemocytes also produce many factors for, and is also mediated in part by, the humoral response (Lavine and Strand, 2002). All haemocytes are derived by a process known as haematopoiesis, and this occurs during two stages of development. The initial group of haemocytes are formed during embryogenesis from mesodermal tissue located at the head or dorsal ends of the insect. These are complemented later by haemocytes produced by hematopoietic organs present during the larval stages of the insect (Lavine and Strand, 2002). Maintenance of the circulating population of these haemocytes is done by the proliferation of cells originally derived in embryogenesis, and the continued release of additional haemocytes from the hematopoietic organs. In Lepidoptera, these organs are located in the meso- and meta-thorax near the imaginal wing discs in pairs. Each of these organs is single-lobed in contrast to the hematopoietic organs in *Drosophila*, which have a primary lobe and several secondary lobes. Similarly to *Drosophila* though, haemocyte numbers within each organ increase during the final instar before metamorphosis occurs (Lavine and Strand, 2002). The number of haemocytes in circulation (i.e. free in the haemolymph) at any time is influenced by the fact that some haemocytes (especially plasmatocytes) may adhere to the tissues. This has not been studied quantitatively. However, it is possible that such tissue adhesion may be affected by the presence of a microbial infection.

Plasmatocytes, along with granular cells make up the majority of the population of circulating haemocytes within *Manduca sexta*. The rest of the population is made up of oenocytoids and spherule cells. Dean et al (2004a, 2004b) has reported the existence of Hyperphagocytic and hyper-spreading haemocytes with in *M. sexta*, although the latter has not been found in uninfected larvae and only seems to appear in response to fungal infection or with injection of laminarin. With the exception of oenocytoids, all these cells are able to proliferate within the hemocoel of the insect.

Prohaemocytes in the hematopoietic organs primarily differentiate into plasmatocytes, whereas those released into the haemocoel differentiate into the other hemocyte classes. As they make up the majority of the hemocyte population it is unsurprising that granular cells and plasmatocytes are the most involved in the cellular response (Lavine and Strand, 2002). Both are able to adhere strongly to foreign surfaces and spread. Granular cells are reported to be the professional phagocytes (i.e. the insect equivalent of macrophages and neutrophils present in mammals), although it has been shown in *M. sexta* that hyperphagocytes actually do most of the phagocytosis (Dean et al., 2004a). Against larger amounts of bacteria, the granular cells and plasmatocytes have been shown to in work in synergy to aggregate the bacteria and encase them in a nodule. It is suggested that oenocytoids also play a role in the melanisation of the nodules in some species.

Photorhabdus luminescens has been shown to inhibit the cellular responses of *M. sexta*. *P. luminescens* strains W14 and TT01 excrete identified and unidentified toxins which inhibit the cellular responses of phagocytosis and nodulation. One identified toxin is LopT which is similar to YopT, a secreted toxin of *Yersinia pestis*, which is delivered into phagocytes via a Type-III Secretion System to prevent phagocytosis (ffrench-Constant et al., 2007a). Other unidentified toxins present in supernatant from cell cultures of *Photorhabdus* have been shown to inhibit the activity of phospholipase A2, responsible for eicosanoid pathway induction and thus aggregation and nodulation of bacteria by haemocytes. Furthermore, co-injection of *P. luminescens* strain W14 supernatant inhibited the phagocytosis of *Escherichia coli* (ffrench-Constant et al., 2007a).

Fluorescent-associated cell sorting (FACS) is a specialised application of flow cytometry with the ability to separate cells on the basis of their fluorescence (Tirouvanziam et al., 2004). This can be very useful for studies on cell populations as it enables the separation of cells according to properties easier to manage. The sample is manipulated so that one cell passes through a laser beam. Depending on the characteristics of the cell, it will scatter some of the light, which is detected by the machine. One detector is in line with the beam and detects the amount of forward scatter (FSC), and several detectors perpendicular to the beam, which measure the amount of side scatter (SSC) as well as any fluorescence from the cell. FSC indicates the size or volume of the cell, while SSC indicates the granularity or complexity of

the cell. The ability to detect fluorescence allows researchers to label the cells with fluorescence markers to enable more accurate identification of cells. After this detection part, a vibrating mechanism is used to create droplets, which contain one cell. This cell is then charged according to the criteria set by the machine and electro-statically deflected into a container.

There are many applications of this technique, and it has been used to study mammalian immunity, however, studies using FACS in insect immunity are few. One such study by Tirouvanziam *et al* (2004) looked at *Drosophila* haemocytes. In this study, the authors were able to label immune related cells and use this to separate out the haemocytes from cell suspension that had been contaminated with yeast cells and cuticular and fat body debris. GSH, an antioxidant essential to functioning immune cells reacts with monochlorobimane (MCB) to produce fluorescent glutathione-*S*-bimane (GSB) adducts. Alternatively, dihydrorhodamine (DHR) reacts with ROS and localises to active mitochrondria. The advantage of the latter method is that together with propidium iodide (PI), only one laser is required to detect both. PI is used to mark dead cells, and thus both of these methods can be used to separate out live haemocytes for further study. Furthermore these haemocytes can be further discriminated by individual reactivity to wheat germ agglutinin (WGA). It was found that plasmatocytes have a low reactivity to WGA while lamellocytes have a high reactivity to WGA. The authors were also able to show a high resolution for reporter genes using FACS. It was demonstrated that there was a five-fold difference in expression in LacZ linked to the *misshapen* gene, part of the Jun kinase cascade, between the top and bottom 20% of lamellocytes. Furthermore, the authors were able to show that despite no increase in size, there was also an increase in WGA binding and intracellular Ca^{2+} levels in the top 20% of cells indicating increased expression. This correlated with reports on mammalian leukocytes.

The aim of this chapter is to investigate the cellular responses of *M. sexta* using FACS following microbial challenges with Gram-negative bacteria, namely *E. coli* and *P. luminescens* strain TT01. Specifically, I asked:

- Is there any change in hemocyte populations in *M. sexta* following immune challenges with *E. coli* and *P. luminescens* strain TT01?

- Are *E. coli* and *P. luminescens* strain TT01 phagocytised by haemocytes?
- Is *E. coli* phagocytised by haemocytes in pre-immunised *M. sexta*?

Results

FACS experiment 1 - Flow cytometry analysis of *Manduca sexta* response to infection from *Escherichia coli* and *Photorhabdus luminescens* strain TT01

To investigate the response of *M. sexta* to bacterial infection, haemocytes were isolated from insects injected with either *E. coli* or *P. luminescens* strain TT01, and incubated with FITC-labelled PNA before flow cytometry analysis.

First for analysis were the controls of insects that remained untreated.

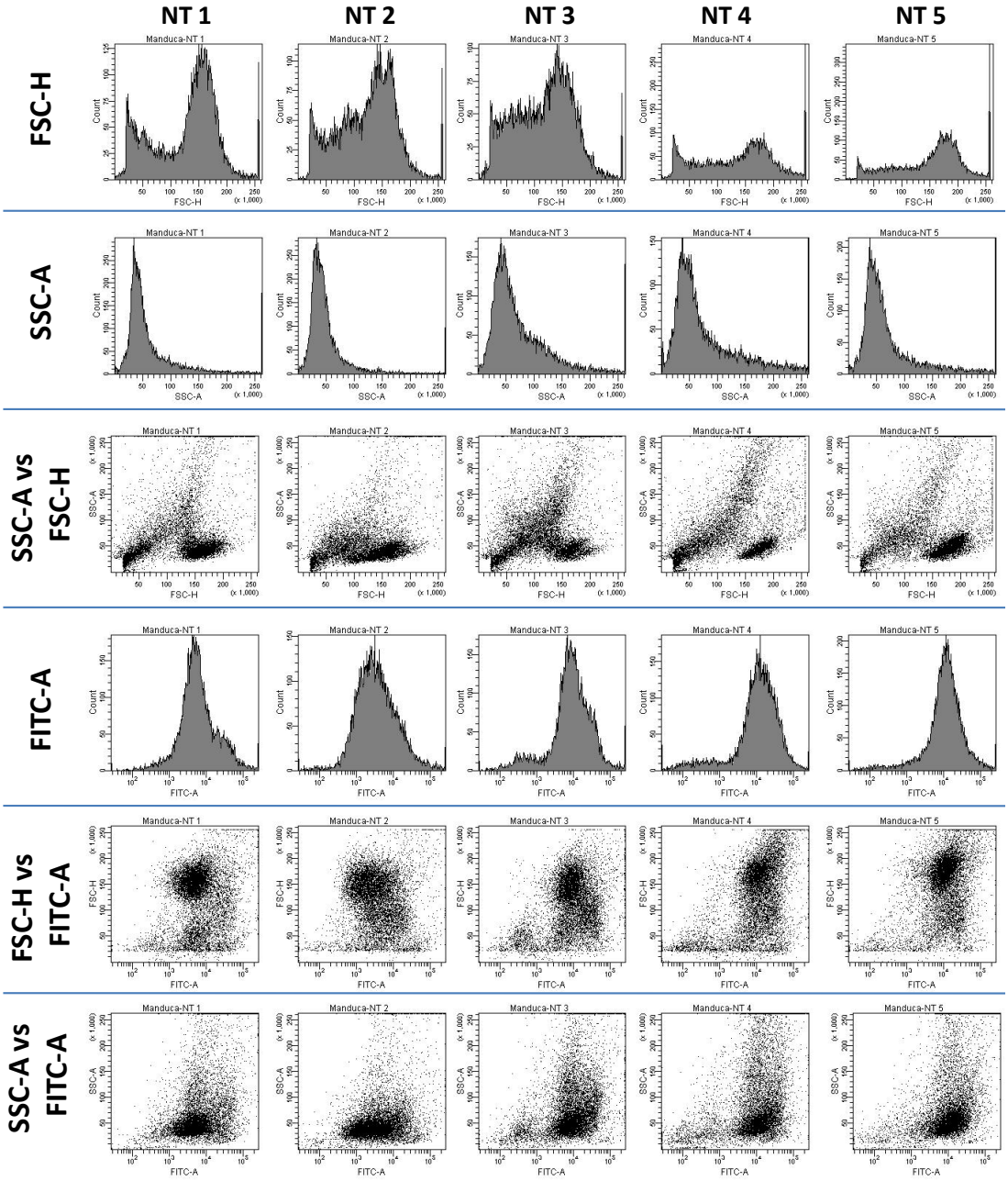

Figure 5.1 – Flow cytometry analysis of untreated (NT) *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects which were incubated with FITC-labelled PNA before flow cytometry analysis. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2-3 groups of cells differing in size but not internal complexity. The PNA bound successfully to cells as shown by the FITC-A histogram . The dotplot of FSC-H vs FITC-A indicates that most of the PNA positive cells come from the group of haemocytes that are bigger in size.

Next for analysis were the controls of insects injected with PBS.

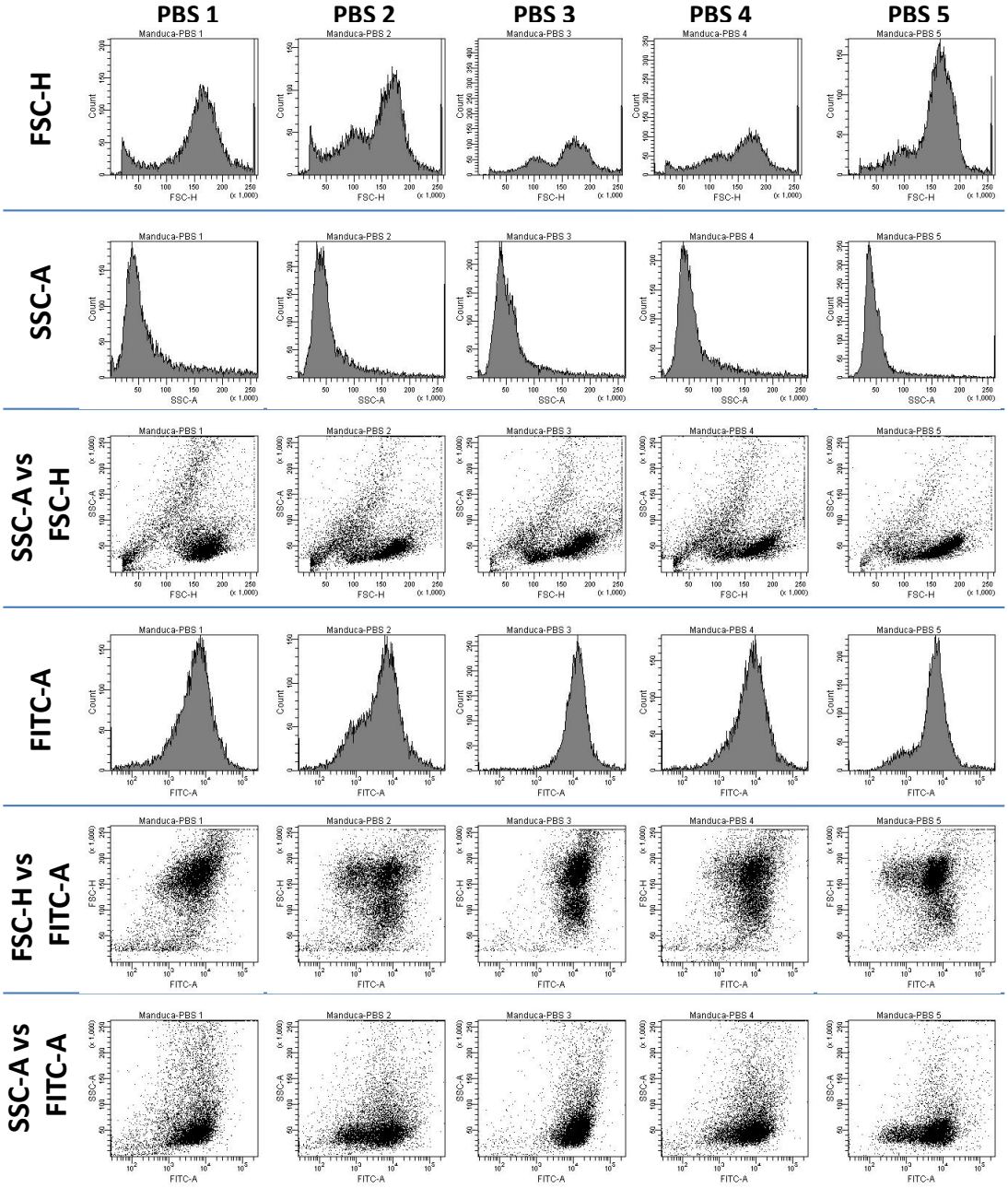

Figure 5.2 – Flow cytometry analysis of PBS injected *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects which were incubated with FITC-labelled PNA before flow cytometry analysis. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. The PNA bound successfully to cells as shown by the FITC-A histogram . The dotplot of FSC-H vs FITC-A indicates that most of the PNA positive cells come from the group of haemocytes that are bigger in size.

Next for analysis were those insects injected with *E. coli*.

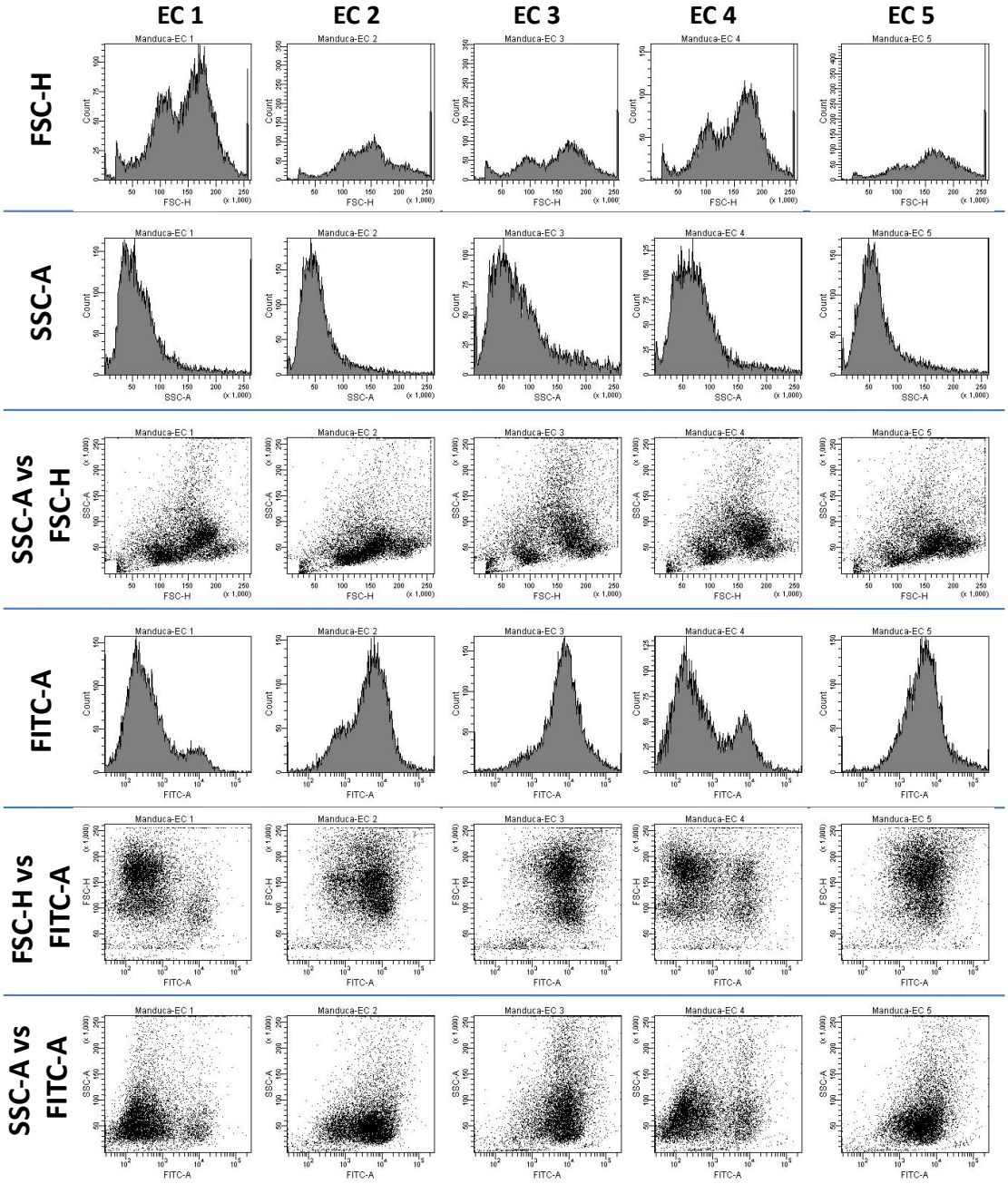

Figure 5.3 – Flow cytometry analysis of *Escherichia coli* injected *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects which were incubated with FITC-labelled PNA before flow cytometry analysis. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size and internal complexity. The PNA bound successfully to some cells as shown by the peak at ~1 x 10^4 on the FITC-A histograms. The dotplot of FSC-H vs FITC-A indicates that most of the PNA positive cells come from the group of haemocytes that are bigger in size.

Finally those insects injected with *P. luminescens* strain TT01 were analysed.

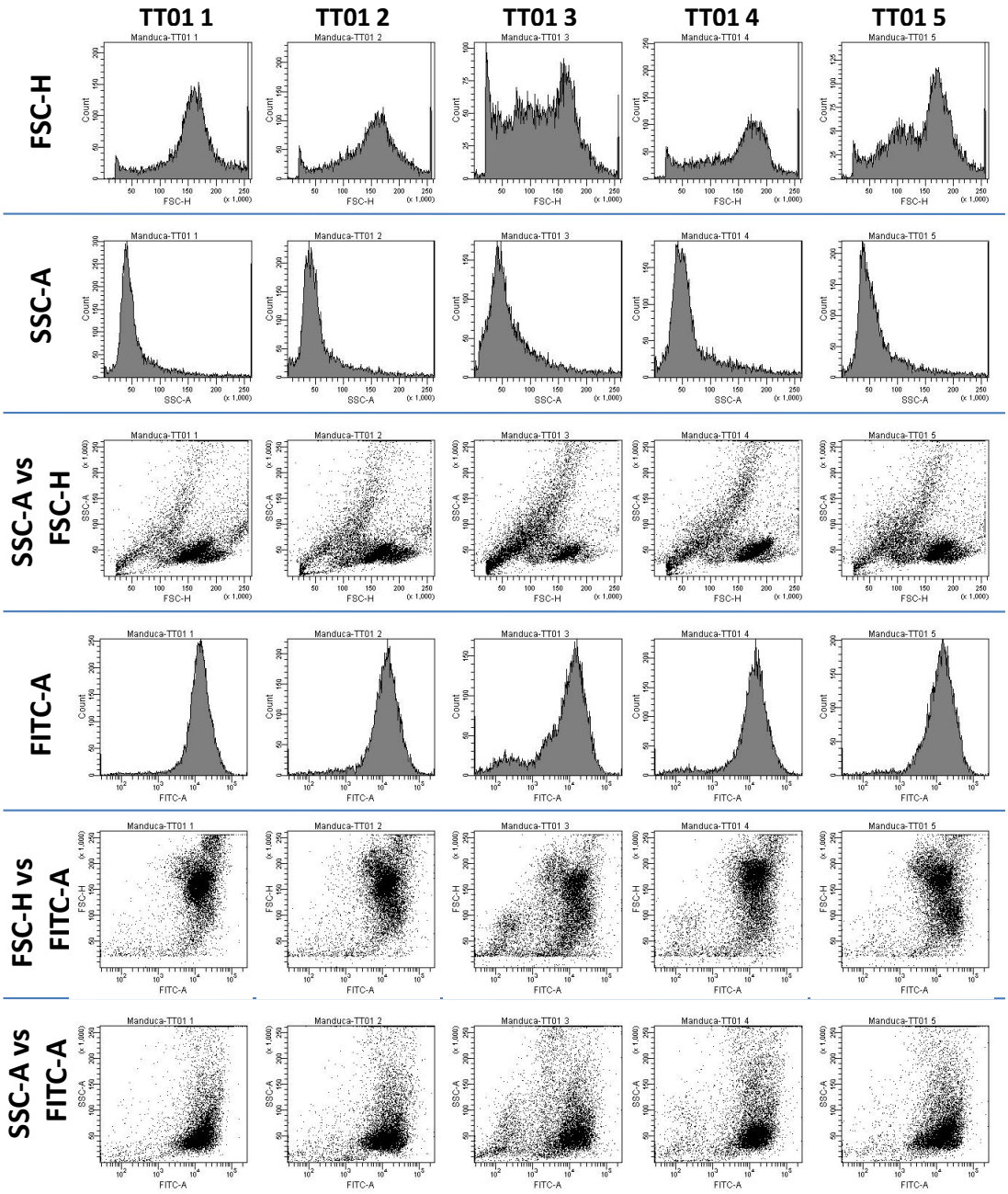

Figure 5.4 – Flow cytometry analysis of *Photorhabdus luminescens* strain TT01 injected *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects which were incubated with FITC-labelled PNA before flow cytometry analysis. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. The PNA bound successfully to cells as shown by the FITC-A histogram . The dotplot of FSC-H vs FITC-A indicates that most of the PNA positive cells come from the group of haemocytes that are bigger in size.

Figures 5.1, 5.2, 5.3 and 5.4 show differently treated insects from FACs experiment 1. Figures 5.1 shows that there are 2 groups of cells that differ in forward scatter (FSC-H) which represents relative size. The FSC-H histograms show one peak that appears at the ~150-175 unit mark on the FSC-H scale representing the major peak in the FSC-H histograms. The other group appears at ~50-100 unit mark on the FSC-H scale. This is seen more clearly in the SSC-A vs FSC-H dotplots. The ratio of the peaks would suggest that most of the cells belong to the group that are larger in relative size. However, the one major peak in side scatter (SSC-A), which represent the granularity/complexity of the cells, histograms suggest that despite a difference in relative size, most share the same level of complexity, with a few cells showing much greater complexity. These observations are confirmed by the SSC-A vs FSC-H dotplots, which again show two groups of cells that differ in relative size but not complexity. The FITC-A histograms are showing the amount of cells that Fluorescein isothiocyanate (FITC) labelled Peanut Agglutinin (PNA) successfully adhered to. This shows one major peak at the ~10^4 unit mark on the FITC-A scale which shows that the PNA successfully adhered to most cells. The FSC-H vs FITC-A and SSC-A vs FITC-A dotplots indicate that the majority of cells that the PNA adhered to were of a larger relative size and of similar complexity although PNA also adhered to a significant amount of cells with greater complexity.

Figure 5.2 represents those insects injected with PBS. This was very similar to the untreated control, which shows that FACS characteristics of the haemocytes are not affected by the injury of a control injection with PBS.

Figure 5.3 represents the insects that were injected with *E. coli*. This shows that although there are still two groups that differ in size, there is an increase the overall granularity/complexity of haemocytes. The major peak in the SSC-A histograms has broadened in all insects injected with *E. coli*, This may be due to the granularity/complexity of the haemocytes rising because they are engulfing the bacteria by phagocytosis. The SSC-A vs FSC-H dotplot indicates that the group of relatively larger cells has increased in granularity/complexity indicating that these may be the cells doing the phagocytosis of the *E. coli*. Overall, there appears to be no change in fluorescence although EC1 and EC4 show a major peak of cells in the FITC-A histograms that have reduced fluorescence. The reason for this is unknown,

perhaps a large group of haemocytes has shed the surface moieties that PNA had successfully adhered to.

Figure 5.4 represents those insects injected with *P. luminescens* strain TT01. This shows no noticeable changes from the two controls. There appear to be two groups of haemocytes that differ in size but not granularity/complexity. This may be due to fewer bacterial cells being injected or that *P. luminescens* strain TT01 prevents it's phagocytosis.

In summary

- There are two main populations of cells. These differ in size but not granularity/complexity in naive/unchallenged insects
- Wounding the insect does not cause any detectable changes in haemocyte populations
- The injection of *E. coli* dramatically increases the granularity/complexity of haemocytes. This is probably due to phagocytosis of the bacteria.
- The injection of *P. luminescens* strain TT01 does not result in any changes of haemocyte population.

Phagocytosis of *Escherichia coli* but not *Photorhabdus luminescens* strain TT01 by *Manduca sexta* haemocytes

To investigate the *in vivo* phagocytosis of bacteria by *M. sexta*, haemocytes were isolated from insects injected with *E. coli* and *P. luminescens* strain TT01. These cells were allowed to form monolayers and then fixed for use in confocal microscopy.

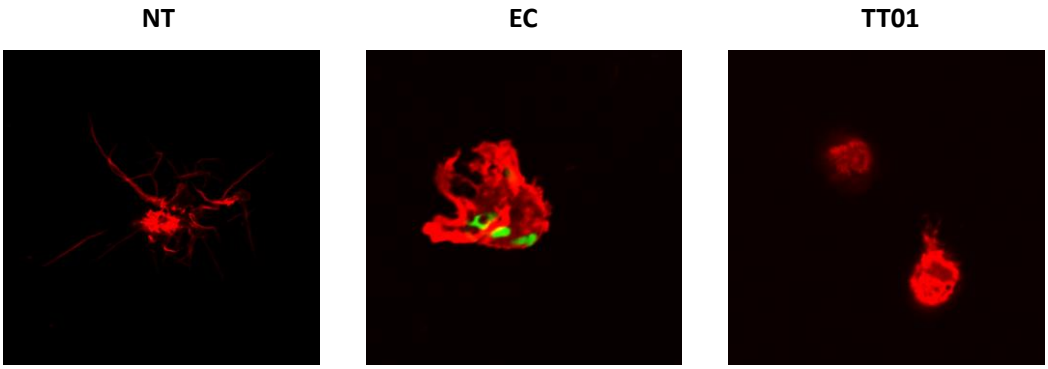

Figure 5.5 – Confocal microscope images of haemocytes from *Manduca sexta* injected with *Escherichia coli* (EC), *Photorhabdus luminescens* strain TT01 (TT01) or left untreated (NT). Panels show haemocytes stained with Texas Red Phalloidin (red) and bacteria expressing GFP (green). Three different insects were used in each treatment, and between 20 -30 cells examined per insect. In insects injected with EC , the bacteria were found inside the haemocytes. With insects that have been injected with TT01, there were no bacteria found inside the haemocytes or present in the sample. In untreated insects, there were no bacteria found in haemocytes or the sample.

As shown by Figure 5.5, phagocytosis of *E. coli* occurs when this bacterium is injected into *M. sexta*, but *P. luminescens* strain TT01 appears to avoid this fate. The haemocytes were stained with Texas-Red conjugated phalloidin and GFP-expressing bacteria were used to appear red and green under confocal microscopy, respectively. The insects that were injected with *E. coli* clearly have the bacteria contained within haemocytes, whereas those insects injected with *P. luminescens* strain TT01 do not appear to have bacteria contained within haemocytes. The untreated control insects also show no GFP-expressing bacteria contained within haemocytes.

FACS Experiment 2 - Flow cytometry analysis of phagocytosis of Green Fluorescent Protein expressing *Escherichia coli* and *Photorhabdus luminescens* strain TT01 by *Manduca sexta*

To further investigate the *in vivo* phagocytosis of bacteria by *M. sexta*, haemocytes were isolated from insects injected with GFP-expressing *E. coli* and *P. luminescens* strain TT01 and used in flow cytometry analysis.

First for analysis were those insects that remained untreated.

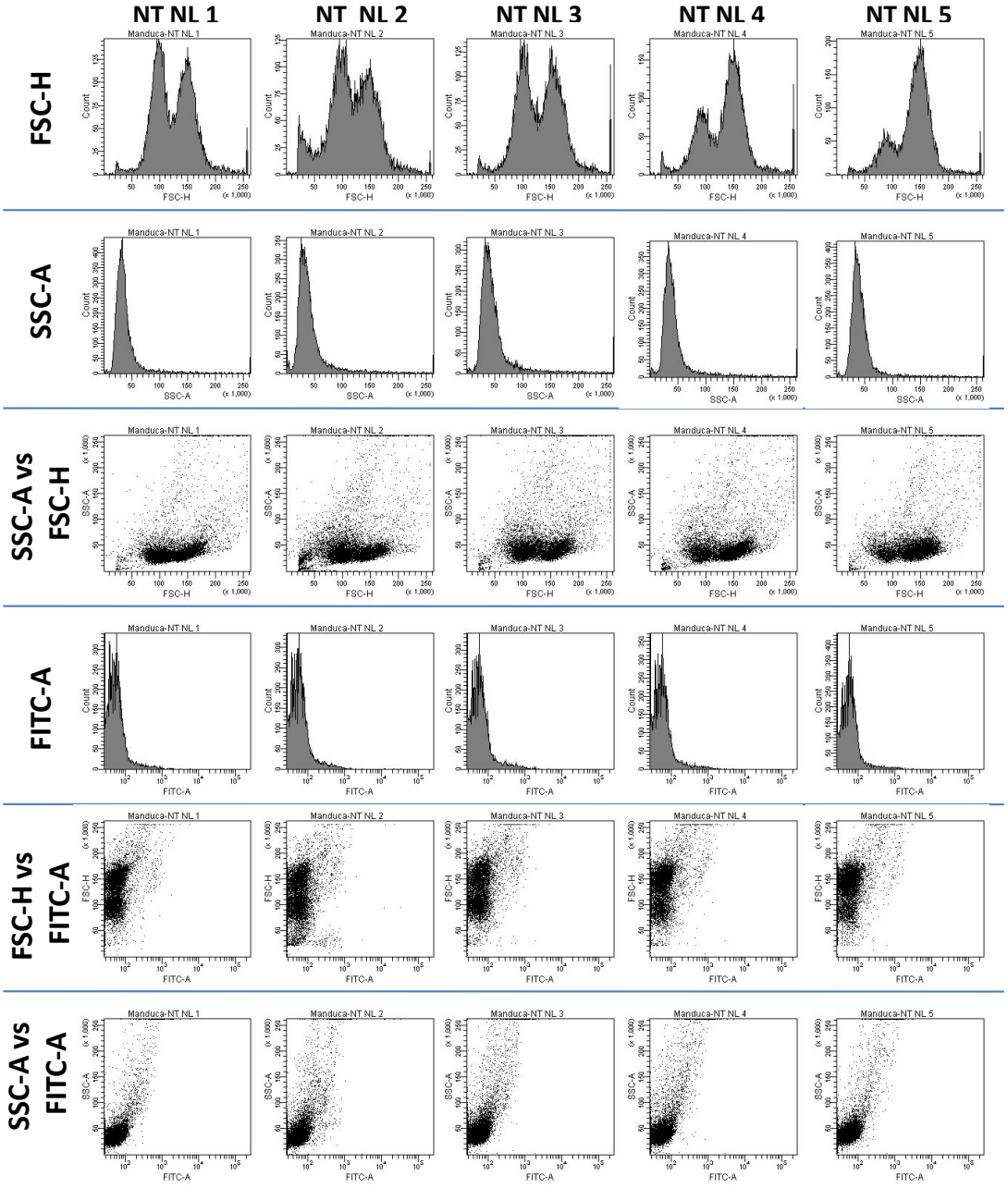

Figure 5.6 – Flow cytometry analysis of untreated *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. The cells are not fluorescent naturally as shown by the peak at 1 $x10^2$ on the FITC-A histogram.

Next for analysis were those insects injected with PBS.

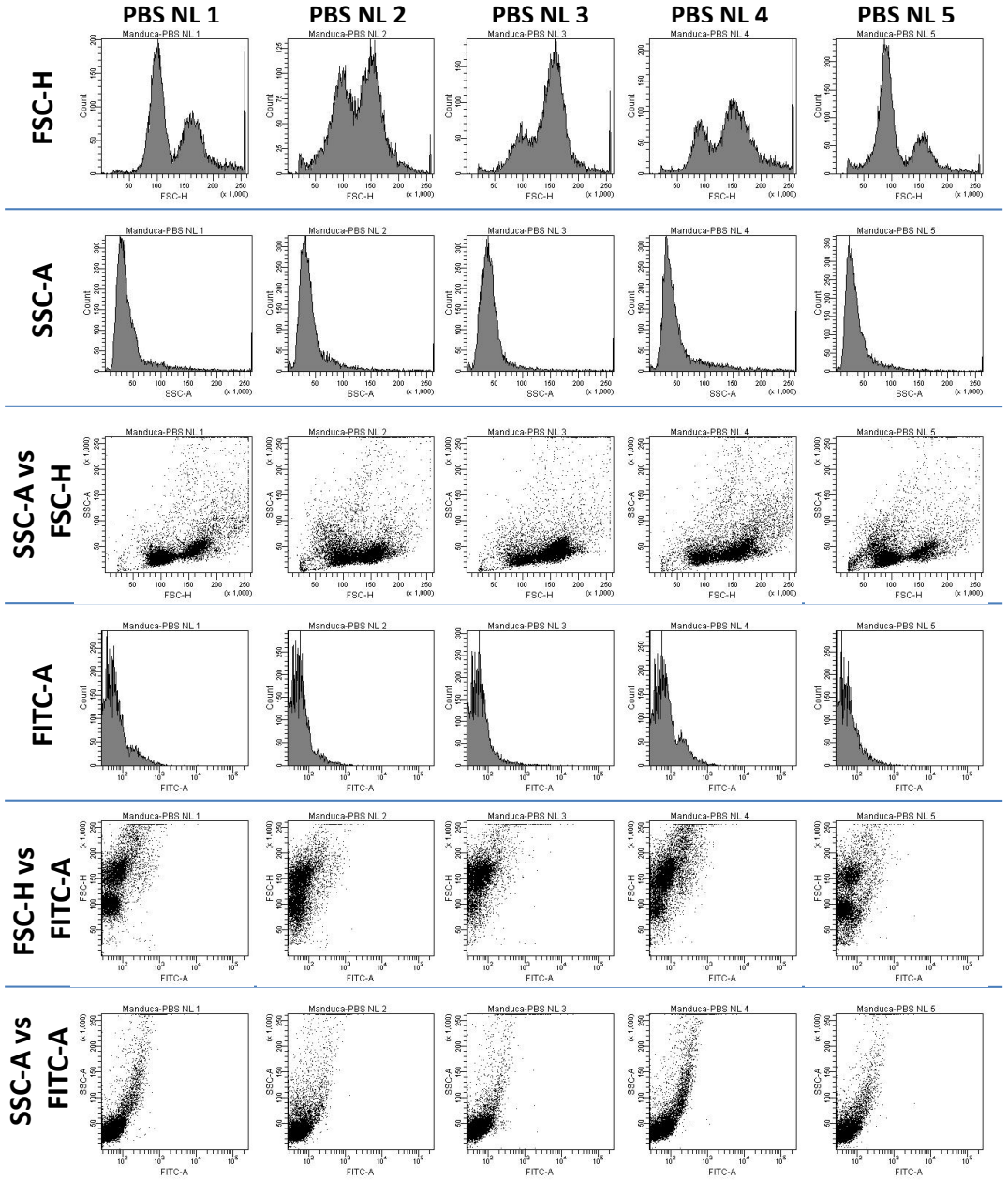

Figure 5.7 – Flow cytometry analysis of PBS injected *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. The cells are not fluorescent naturally as shown by the peak at 1 $x10^2$ on the FITC-A histogram.

Next for analysis were those insects injected with GFP-expressing *E. coli.*

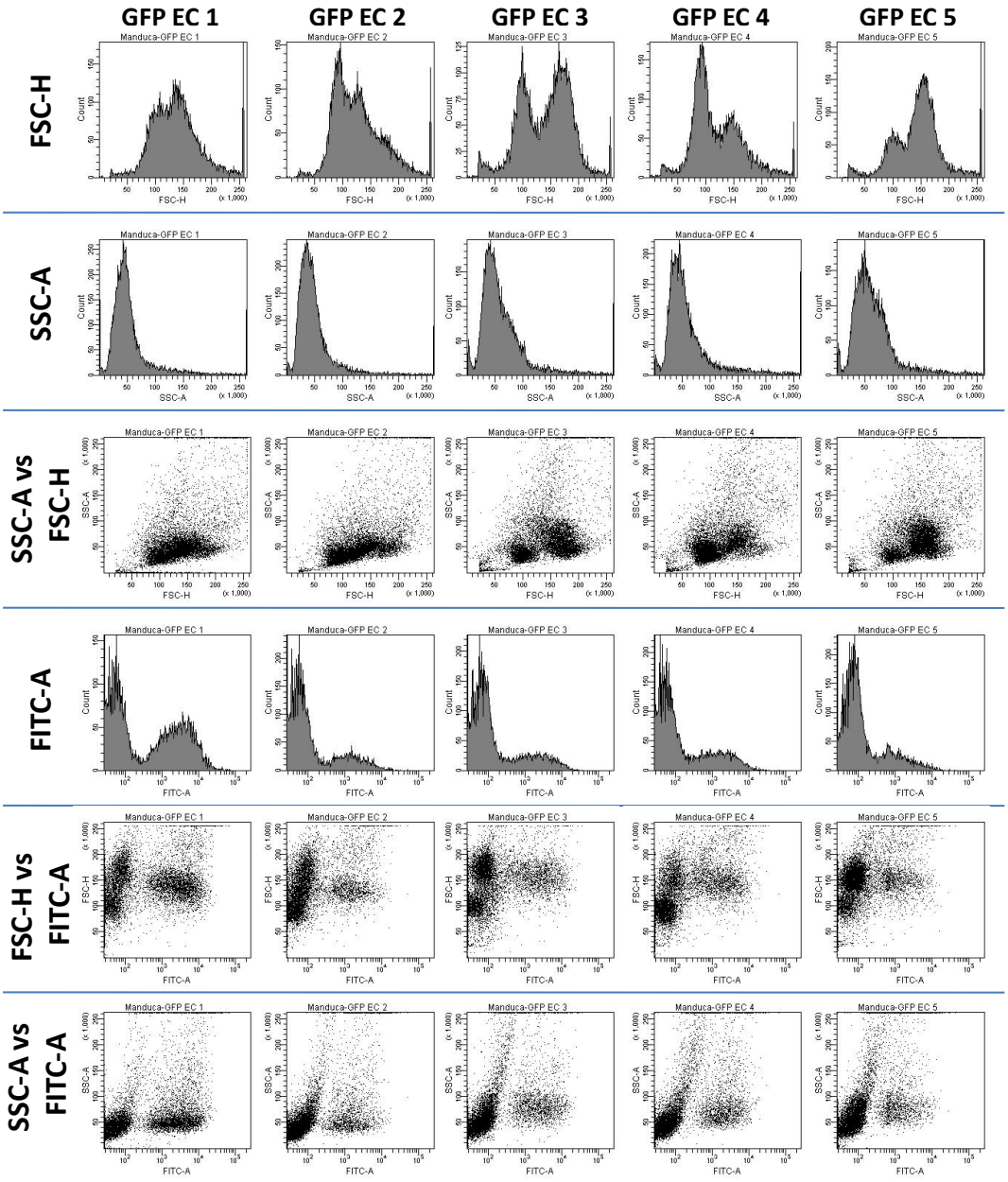

Figure 5.8 – Flow cytometry analysis of GFP-expressing *Escherichia coli* injected *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size and internal complexity. Most of the cells are not fluorescent naturally as shown by the major peak at 1 x10^2 on the FITC-A histogram, but a minor peak at 1 x 10^3 indicates that some cells are more fluorescent than normal. Plotting FSC-H vs FITC-H reveals that the fluorescent cells are mainly from the ~150FSC-H group. Plotting SSC-A vs FITC-A shows that the fluorescent cells are slightly more complex than the majority of cells, measuring ~75 SSC-A units.

Last for analysis were those insects injected with GFP-expressing *P. luminescens* strain TT01.

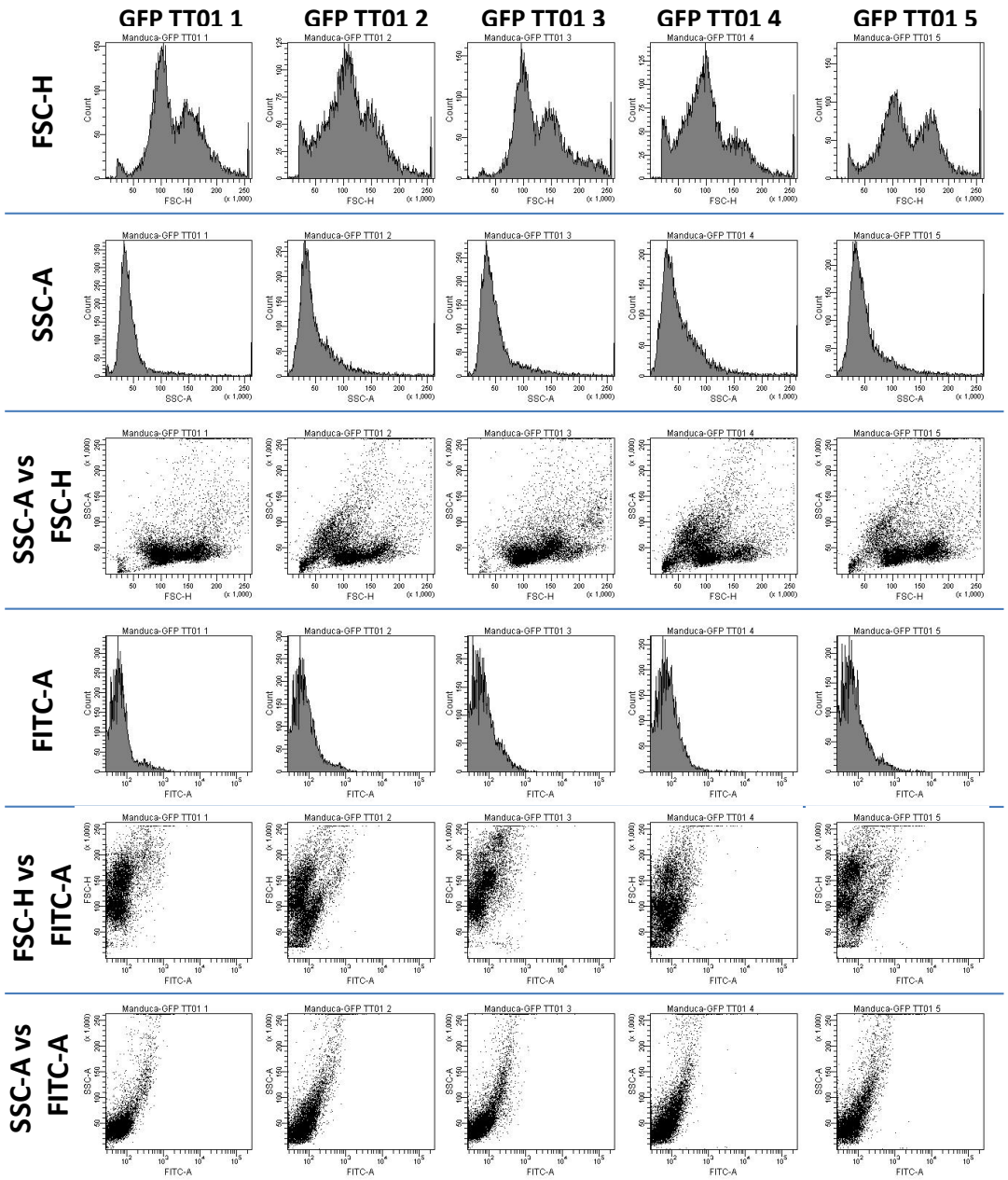

Figure 5.9 – Flow cytometry analysis of GFP-expressing *Photorhabdus luminescens* strain TT01 injected *Manduca sexta* showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. The cells are not fluorescent naturally as shown by the peak at 1 $x10^2$ on the FITC-A histogram.

Figures 5.6, 5.7, 5.8 and 5.9 show the results form FACS experiment 2. Figure 5.6 shows a change from what was previously seen in Figure 5.1, in that there are two distinct peaks of higher and lower FSC-H values, about ~150 and ~100 FSC-H units respectively. Moreover, the two groups that seemed to appear in the dotplot of SSC-A vs FSC-H in Figure 5.1 have merged somewhat, perhaps representing considerable overlap between the two groups that were seen in the last set of FACS experiments. The lack of complexity remains the same though, with most haemocytes sharing the same low level and a few that have a rising level of granularity. The major peak at a low fluorescence on the FITC-A histograms indicate that the haemocytes have a low level of natural fluorescence.

Figure 5.7 shows that again the PBS treatment is very similar to the untreated control. The two groups of cells differing in relative size might be a bit more distinct perhaps but this will be down to variation between insect individuals rather that any effect of injecting PBS. Also, this treatment shows that PBS does not make the haemocytes fluorescent, as there is no change in fluorescence from the untreated control.

Figure 5.8 shows that the injection of GFP-expressing *E. coli* increases the complexity of the cells with the SSC-A histograms showing a broader peak than what was seen in Figures 5.6 and 5.7. Similarly to Figure 5.3, there is an increase of granularity from the cells with a larger relative size. The FITC-A histograms, in contrast to Figure 5.6 and 5.7, show a peak of cells that exhibit fluorescence. This indicates that some cells have managed to engulf the GFP-expressing *E. coli* by phagocytosis. The FSC-H vs FITC-A dotplots throw further light on to the situation by revealing that most of the fluorescent cells are larger in relative size. This may be due though to the haemocyte increasing in size to accommodate the bacteria inside.

Figure 5.9 shows a similar story to controls. There is little difference in complexity of the haemocytes, indicating that no phagocytosis of *P. luminescens* strain TT01 has occurred. The lack of a higher fluorescent peak in the FITC-A histograms confirms this.

In summary,

- The two groups previously seen in FACS experiment 1, seem to have merged into one group although there are still two distinct peaks seen in controls. Some overlap between the two groups has probably occurred.
- Despite the merging of the groups, most haemocytes don't show much complexity.
- Injection of GFP-expressing *E. coli* increases the granularity/complexity of the haemocytes.
- Fluorescence peak shows that this increased granularity/complexity is due to the phagocytosis of GFP-expressing *E.coli*.
- Haemocytes of a larger relative size are found to have the fluorescent bacteria inside them, although the phagocytosis of bacteria probably causes an increase in size.
- There appears to be no phagocytosis of GFP-expressing *P. luminescens* strain TT01

FACS Experiment 3 - Flow cytometry analysis of phagocytosis of Green Fluorescent Protein expressing *Escherichia coli* by *Manduca sexta* pre-treated with bacteria

To investigate phagocytosis of bacteria by *M. sexta* that had been previously exposed to bacteria, insects were injected with either *E. coli* or *P. luminescens* (or a control treatment); after 18 hours these insects were injected with GFP E. coli, and 1 hour later haemocytes were isolated in the usual way. These samples were then used in flow cytometry analysis.

First for analysis were those insects that were untreated before injection with GFP-expressing *E. coli*.

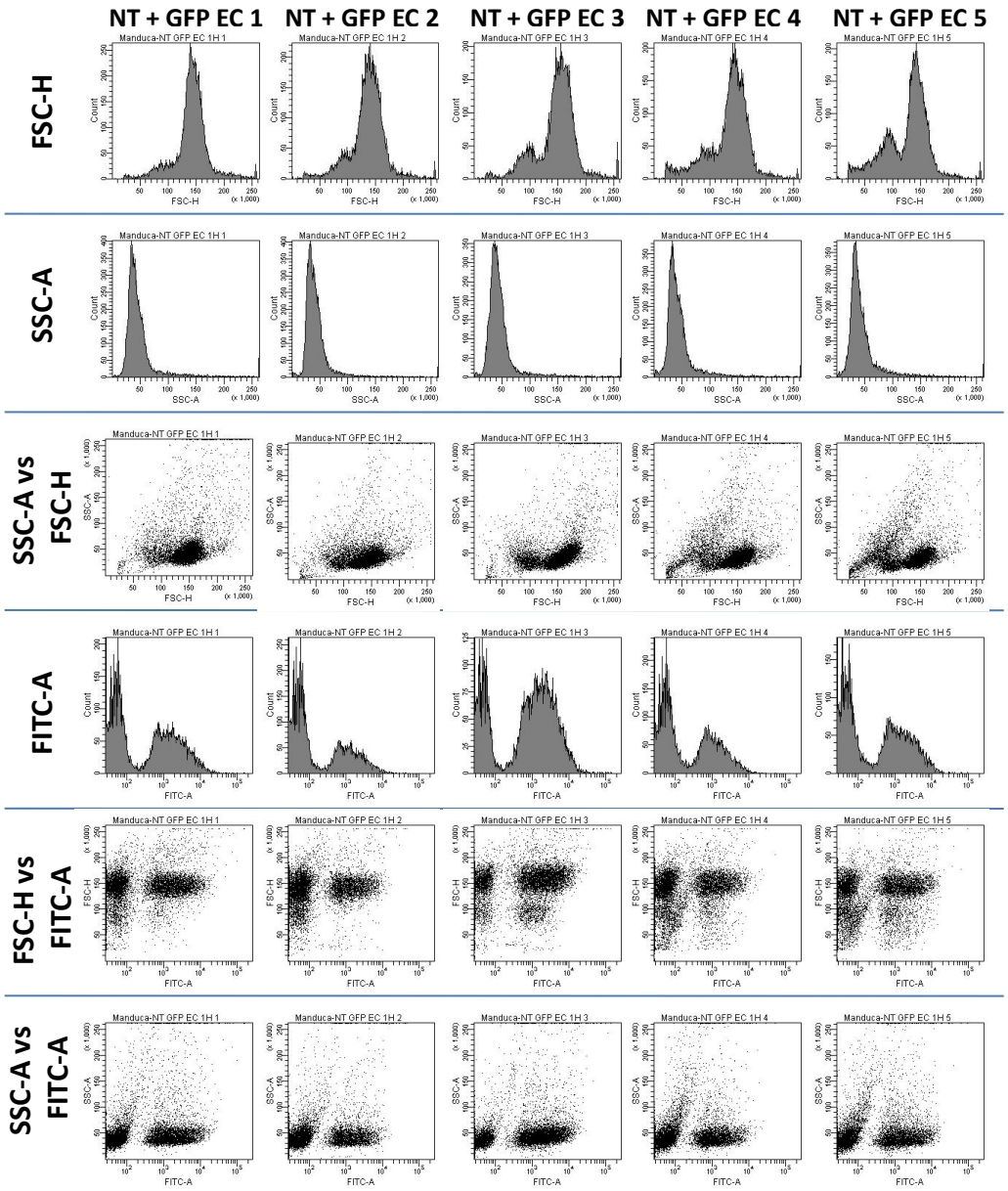

Figure 5.10 – Flow cytometry analysis of GFP-expressing *Escherichia coli* injected *Manduca sexta* that was not pre-immunised, showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of hemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. There are two groups of cells that differ in fluorescence as shown by the two peaks on the FITC-A histogram. Plotting FSC-H vs FITC-A shows that the majority of these more fluorescent cells come from the ~150 FSC-H group. Plotting SSC-A vs FITC-A reveals that the higher fluorescing cells have similar complexity to the lower fluorescing cells.

Next for analysis were those insects pre-immunised with PBS before injection with GFP-expressing *E. coli*.

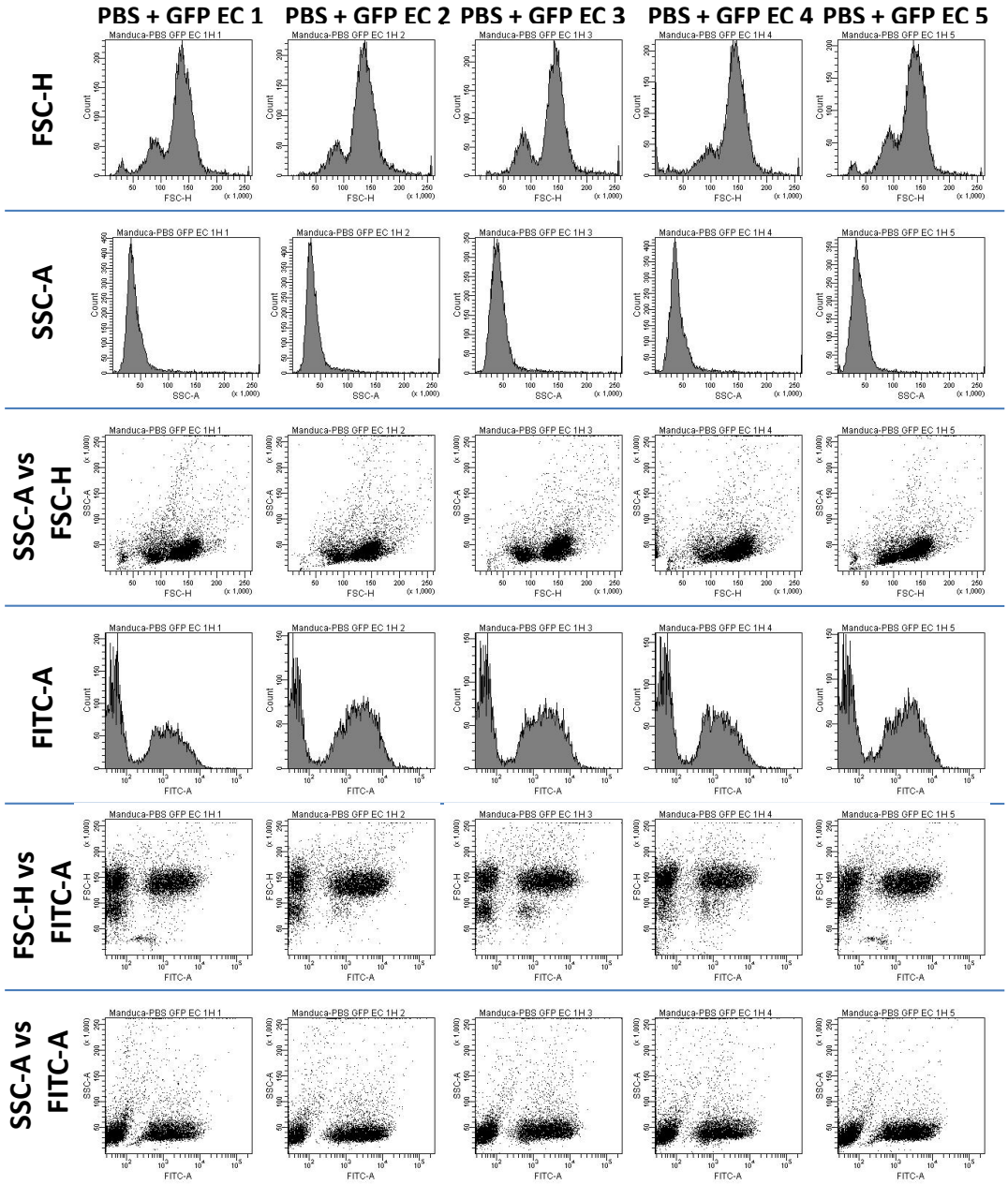

Figure 5.11 – Flow cytometry analysis of GFP-expressing *Escherichia coli* injected *Manduca sexta* that were pre-immunised with PBS, showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size but not internal complexity. There are two groups of cells that differ in fluorescence as shown by the two peaks on the FITC-A histogram. Plotting FSC-H vs FITC-A shows that the majority of these more fluorescent cells come from the ~150 FSC-H group. Plotting SSC-A vs FITC-A reveals that the higher fluorescing cells have similar complexity to the lower fluorescing cells.

Next for analysis were those insects pre-immunised with *E. coli* before injection with GFP-expressing *E. coli*.

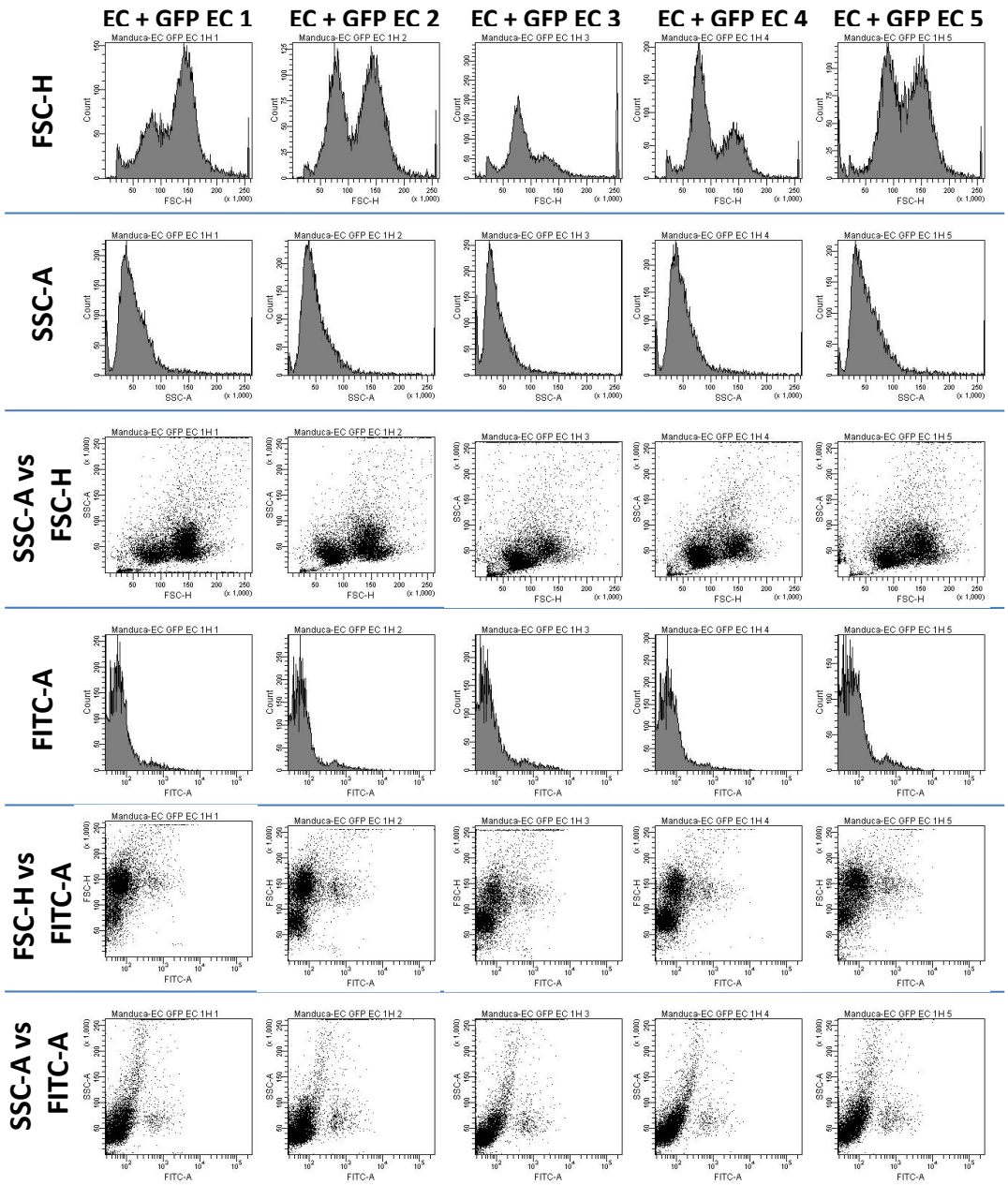

Figure 5.12 – Flow cytometry analysis of GFP-expressing *Escherichia coli* injected *Manduca sexta* that were pre-immunised with *E. coli*, showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size and internal complexity. There is one major peak at 1 x 10^2 on the FITC-A histogram indicating that the cells are not naturally fluorescent but there is a 'shoulder' at ~1 x 10^3 which suggest that a small number of cells have a higher fluorescence than normal.

Lastly, those insects pre-immunised with *P. luminescens* strain TT01 before injection with GFP-expressing *E. coli* were analysed.

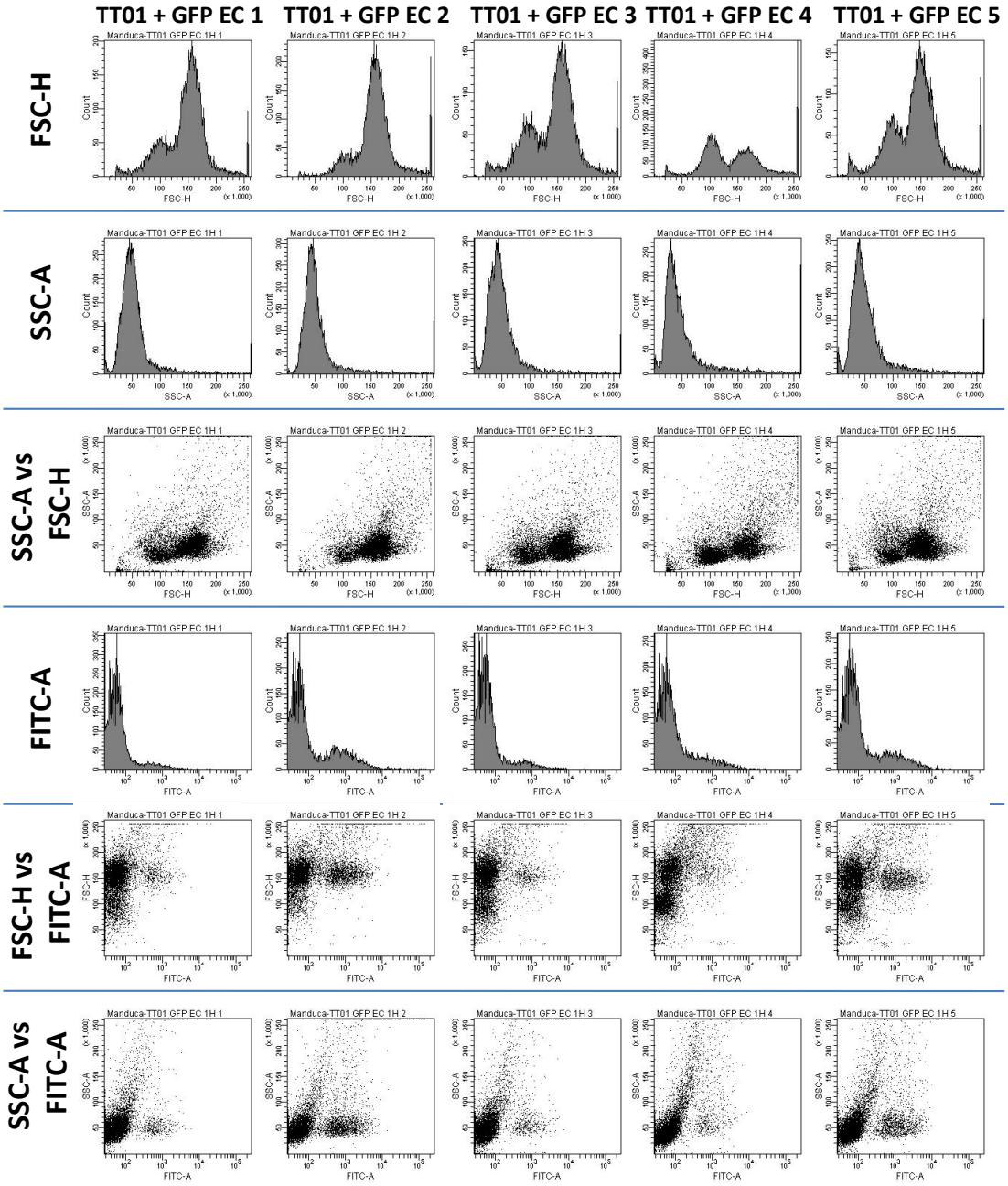

Figure 5.13 – Flow cytometry analysis of GFP-expressing *Escherichia coli* injected *Manduca sexta* that were pre-immunised with *Photorhabdus luminescens* strain TT01, showing forward scatter (FSC-H = relative size), side scatter (SSC-A = complexity) and fluorescence (FITC-A) of haemocytes. Each row of five panels shows cell counts from different similarly treated insects. The FSC-H and SSC-A histograms along with the FSC-H vs SSC-A dotplot appear to show 2 groups of cells differing in size and internal complexity. There is one major peak at 1 x 10^2 on the FITC-A histogram indicating that the cells are not naturally fluorescent but there is a 'shoulder' at ~1 x 10^3 which suggest that a small number of cells have a higher fluorescence than normal.

Figures 5.10, 5.11, 5.12 and 5.13 show results from FACS experiment 3. Figure 5.10 shows only one major peak in the FSC-H histograms, indicating that most haemocytes are of a similar size. Also, the SSC-A histograms show a narrow peak, despite injection with GFP-expressing *E. coli*. This is probably due to the fact that it is only a very short infection, and so many haemocytes haven't had much time to gain complexity by engulfing bacteria. The higher fluorescent peak indicates that phagocytosis is occurring and the FSC-H vs FITC-A dotplot indicates that cells with relatively larger sizes is doing most of phagocytosis, although, with these are making up most of the population, it is not unexpected. Figure 5.12 tells a similar story.

Figure 5.12 shows more of a difference though. There are two distinct peaks indicating two groups of cells. The SSC-A histograms show an increase in complexity as a result of the first injection of *E. coli*. However, the FITC-A shows no higher fluorescent peak, so there is little phagocytosis of the GFP-expressing bacteria, much less than in naive insects. This could be that the haemocytes are already full of bacteria and thus aren't engulfing any more, or the AMP response is killing the GFP-expressing bacteria before phagocytosis can occur.

Figure 5.13 shows more similarity to the controls. There appears to be only one major group of haemocytes, but the complexity appears a little greater than either the controls. In contrast to the controls, there only appears to a small peak of higher fluorescence present in the FITC-A histograms, although it is larger than what appears in Figure 5.12. It indicates that *P. luminescens* strain TT01 is preventing phagocytosis of GFP-expressing *E. coli*.

In summary,

- In naive insects there is one major group of cells with low complexity/granularity despite being injected with GFP-expressing *E. coli*
- There is phagocytosis occurring of GFP-expressing bacteria in naive insects
- Insects pre-treated with *E. coli* show little or no phagocytosis of GFP-expressing *E. coli*. Is this due to haemocytes being full or increased expression of AMPs

- Insects pre-treated with *P. luminescens* strain TT01 also show little phagocytosis of GFP-expressing bacteria. This is probably due to *Photorhabdus* preventing phagocytosis.

Cell-free plasma from pre-treated *Manduca sexta* kills Green Fluorescent Protein expressing *Escherichia coli*

To investigate the ability of cell-free plasma to kill GFP-expressing *E.coli in vitro,* plasma was isolated from *M. sexta* pre-treated with either *E. coli* or *P. luminescens* strain TT01 and the cells removed by centrifugation. GFP-expressing *E. coli* were added to the cell-free plasma and incubated for one hour before being spread on selective plates of LB.

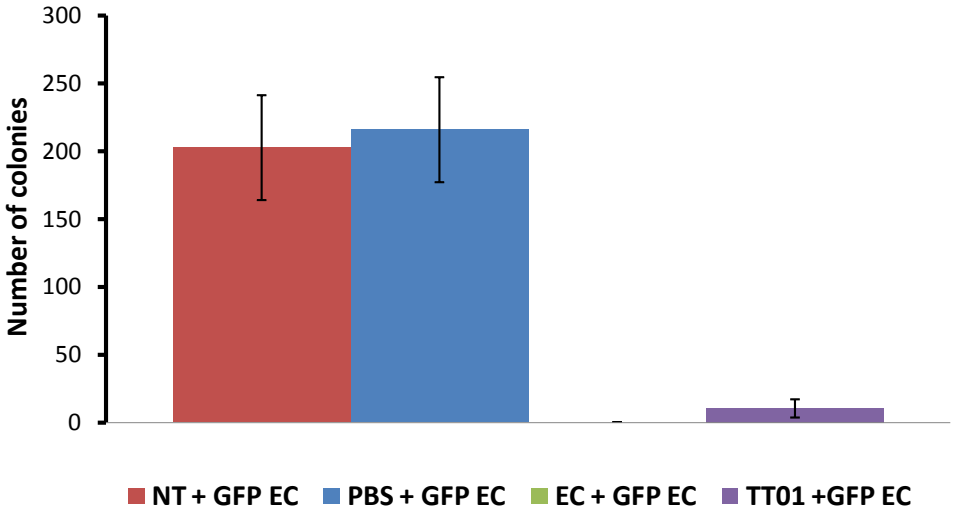

Figure 5.14 – The number of Green Fluorescent Protein expressing *Escherichia coli* colonies present on selective media after incubation in cell-free plasma taken from pre-immunised *Manduca sexta*. The columns represent mean values ± standard deviation (n=5 repeats) 24 hours after plating on selective media. There was little or no growth of GFP-expressing *E. coli* in cell-free plasma taken from insects pre-immunised with either *Photorhabdus luminescens* strain TT01 or *E. coli* respectively whereas growth in cell-free plasma from controls of insects pre-immunised with PBS or left untreated, was uninhibited.

As shown in Figure 5.14, GFP-expressing *E. coli* are unable to survive in cell-free plasma of those insects that have been pre-treated with bacteria. When incubated in cell-free plasma taken from controls of insects, that been injected with PBS or left untreated, GFP-expressing *E. coli* were able to survive, resulting in ~200 colonies growing on selective media. However, when GFP-expressing *E. coli* was incubated

in cell-free plasma taken from insects pre-treated with *E. coli*, and plated on to selective media, no colonies grew. Similarly when GFP-expressing *E. coli* were incubated in cell-free plasma, taken from insects pre-treated with *P. luminescens* strain TT01 and plated on to selective media, very few colonies grew (~10).

Discussion

The main findings of this chapter are as follows:

- There was an increase in the granularity/complexity of haemocytes following infection with *E. coli.* There were no major changes in haemocyte populations towards *P. luminescens* strain TT01.
- *P. luminescens* strain TT01 was not found to be phagocytised by insect haemocytes.
- Phagocytosis of GFP *E. coli* by bacterially pre-treated *M. sexta* was less than naive insects.
- Pre-immunisation with *P. luminescens* strain TT01 results in no phagocytosis of GFP-expressing *E. coli*.
- There was little or no recovery of GFP *E. coli* after incubation in cell-free plasma taken from bacterially pre-treated *M. sexta*.

In contrast to humoral responses, there is little known about the cellular response (Nardi et al., 2003), although much has been done recently to reverse this trend. As described above, FACS can be an extremely useful technique to aid research into this particular area. Here I attempted to use FACS to reveal a little more of the cellular response.

There was an increase in the granularity/complexity of haemocytes in response to *E. coli* infection, but there no major changes detected in the haemocyte population in response to *P. luminescens* strain TT01 infection. PNA binds to all activated haemocytes; this includes plasmatocytes and granular cells, which make up the majority of haemocytes found in *M. sexta*. Most of the control insects; untreated and injected with PBS, show one major peak of fluorescence (Figure 5.1, Figure 5.2). It would be expected that there would be two peaks of fluorescence, one low and one high, indeed future experiments done with no PNA, show that the haemocytes

naturally have a low fluorescence (Figure 5.6, Figure 5.7). Similarly, those insects injected with *P. luminescens* strain TT01 also show one peak, although this should be expected (Figure 5.4). Those insects injected with *E. coli*, however, show a lot of variation between individual insects, as shown in Figure 5.3 there are a couple of peaks in samples EC1 and EC4 that show low fluorescence. This may represent new cells (plasmatocytes) that are un-activated, or maybe were unlabelled in the first place. It is possible that my experimental technique with a new protocol may be at fault for these results. However, the use of PNA within the protocol should perhaps be changed to either specific antibodies for individual haemocytes or to another lectin. PNA does bind only to granular cells *in situ* (Nardi et al., 2003) but when activated release PNA binding proteins to activate other haemocytes including plasmatocytes (Nardi - personal communication). It is probable that these cells were activated during the experimental protocol, as the cells would have come into contact with foreign surfaces.

Although the experiment did not go quite as planned, it was interesting to note that side scatter, an indicator of the granularity/complexity of cells was overall much less in *P. luminescens* strain TT01 infections than it was in *E. coli* infections (Figures 5.3, 5.4, 5.8, 5.9, 5.12, 5.13). As described above, *P. luminescens* employs a variety of effectors to prevent phagocytosis. The confocal microscopy results (Figure 5.5) adds to the current evidence that this is the case as no cells were found with *P. luminescens* strain TT01 inside. It also proved difficult to find the cells on the slide, suggesting that haemocytes numbers are also reduced as a result of the infection.

FACS experiment 2 also confirms the lack of phagocytosis of *P. luminescens* strain TT01. Experiments done with GFP-expressing bacteria show that *E. coli* is phagocytised by haemocytes, while *P. luminescens* strain TT01 is not. Figure 5.8 shows a peak of higher fluorescence than what is normally found in naive insects (Figure 5.6, Figure 5.7). The other graphs within Figure 5.8 confirm that the peak is due to a group of cells that measure ~150 units on the FSC-H scale, which is too big to be bacterial cells. Figure 5.9, however shows no peak of higher fluorescence, indicating that no phagocytosis has taken place. However it should be noted that far fewer *P. luminescens* strain TT01 cells were injected than *E. coli* cells and thus skews the experiment towards finding *E. coli* cells. To ensure a fair comparison, an equal number of cells should be injected.

Further evidence for inhibitory factors of phagocytosis secreted by *P. luminescens* strain TT01 is provided by the next set of experiments. Phagocytosis of GFP-expressing *E. coli* following pre-treatment of *M. sexta* with *P. luminescens* strain TT01 is much less than in controls (Figure 5.9, Figure 5.10 and Figure 5.13). However, pre-treatment with *E. coli* resulted in even less phagocytosis of GFP-expressing *E. coli* (Figure 5.12). The reason for this is unclear, the *E. coli* strain used here is not expressing any inhibitory factors, as proved earlier (Figure 5.8). So the lack of phagocytosis could be due to two reasons; firstly, the phagocytes are full, and no new phagocytes have differentiated yet, or secondly, the humoral response is killing the bacteria before they come into contact with the haemocytes. This second point could also explain the *P. luminescens* strain TT01 result as well.

The final experiment (Figure 5.14) indicates that the second point is the case. Colony forming units (CFU) of GFP-expressing *E. coli* could not be recovered from cell-free plasma taken from *M. sexta* pre-treated with *E. coli*. Very few CFU were recovered from cell-free plasma those pre-treated with *P. luminescens* strain TT01. In contrast, the controls had many CFU recovered. This indicates that the activated humoral response is responsible for the lack of phagocytosis.

It is clear that a lot of work still need to be done to elucidate the role of haemocytes in the immune response. Specific antibodies for markers of haemocytes would need to be used to investigate changes in hemocyte populations in response to infection. These markers would also help to identify the role of different hemocyte types during infection. Also using a different pathogen or knock-out mutants of *P. luminescens* strain TT01 would help to understand the infection process and the immune response a little better.

Chapter 6 – Discussion

The pathogenesis of *Photorhabdus luminescens* strain TT01 against the immune defence of *Manduca sexta* is similar to an arms race whereby each organism is trying to kill each other first. It is a race though, that immunologically naive insects often lose. *P. luminescens* strain TT01 employs a range of effectors that inhibit the immune responses of *M. sexta* including phagocytosis, nodule formation and pro-phenoloxidase (PPO) activation and produces a range of toxins that cause cell death, ultimately proving too much for the caterpillar. However, should *M. sexta* be pre-treated with a harmless bacterium, in this case, *Escherichia coli*, then the insect is usually able to survive a *P. luminescens* strain TT01 infection (Eleftherianos et al., 2006a), *E. coli* effectively pre-arms the caterpillar against attack.

An important first step of any immune defence is the ability to recognise the presence of pathogens within the host. *M. sexta*, like many organisms produces a range of PRRs to recognise MAMPs and initiate immune defences. One such PRR is peptidoglycan recognition protein (PGRP) and it's role in the immune defence of *M. sexta* was studied in chapter three. PGRP recognises peptidoglycan (PGN) and it is thought that it plays a role in up-regulating the expression of antimicrobial peptides (AMPs) (Kanost et al., 2004). The PGRP system within *Drosophila* is able to discriminate between Gram-negative and Gram-positive bacteria and initiate an appropriate response through activation of either the Toll or *IMD* pathway (Lemaitre and Hoffmann, 2007). There is no evidence that the *M. sexta* is able to discriminate in a similar way. Results in chapter three show that PGRP mRNA and protein levels are up-regulated, in response to infection with *E. coli* and *P. luminescens* strain TT01, both Gram-negative bacteria (Figure 3.1, Figure 3.2). The response to *E. coli* is transient but quick with seemingly maximal levels of PGRP mRNA at four hours after infection (Figure 3.3). It is clear that *M. sexta* is able to recognise the presence of *P. luminescens* strain TT01 within its body.

Two further PRRs have been shown to be up-regulated following infection with *P. luminescens* strain TT01; hemolin and immulectin-2 (IML-2) (Eleftherianos et al., 2006a, Eleftherianos et al., 2006b). Both bind to lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria cell walls. The knock down of any of these PRRs (PGRP, hemolin and IML-2) by RNA interference (RNAi), results in *M. sexta*

becoming more susceptible to *P. luminescens* strain TT01 infection, with or without the pre-immunising effect of *E. coli* (Figure 3.6). This is unexpected as it would be thought that there would be a level of redundancy within the system, and that the insect would be able to cope despite the loss of one PRR.

RNAi is a powerful technique that allows the knock down of a specific gene through the recognition and degradation of the mRNA transcript for that gene. The technique is based upon the organism's natural defences against viral RNA (Eleftherianos et al., 2006a, , 2006b). Many viruses contain a double-stranded RNA (dsRNA) genome, and so organisms have developed through natural selection a mechanism by which to degrade any dsRNA molecules found within cells. Researchers can take advantage of this mechanism by introducing dsRNA analogous to the mRNA transcript of the gene of interest and the result should be the knock down in expression of this particular gene through the degradation of the mRNA transcript. The process is mediated by a complex of proteins known as RISC, which when presented with a template of RNA will seek out analogous mRNA molecules and cleave them. The process is outlined in Figure 6.1.

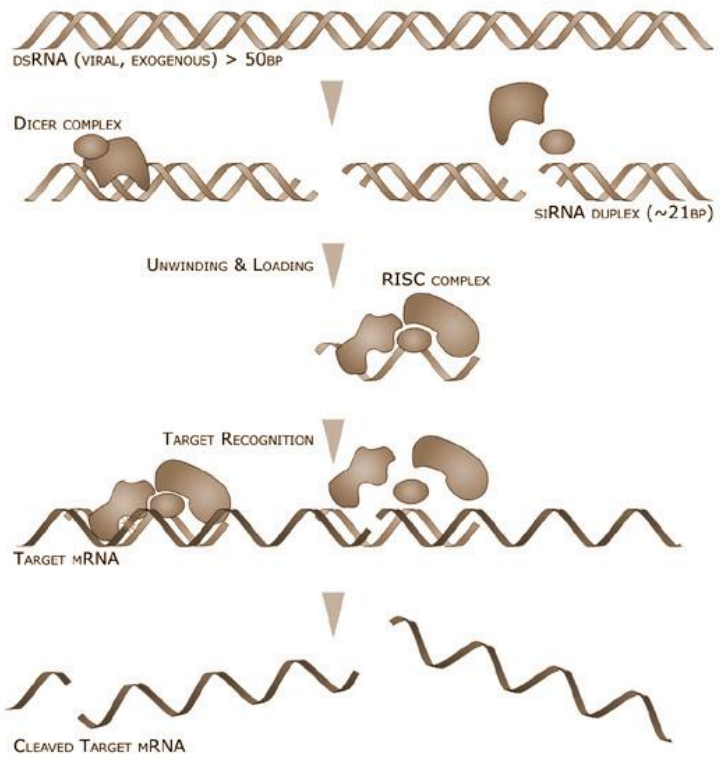

Figure 6.1 – Overview of the mechanism of RNA interference (http://www.mekentosj.com/irnai/rnai.html)

The fact that the knock down of each PRR is so detrimental to survival of the insect suggests that each one has a specific role within the immune system and all are required to initiate the different pathways. Hemolin has been shown to mediate the cellular response, with its knock down associated with reduced phagocytosis and formation of melanotic nodules in response to challenge with *E. coli* (Eleftherianos et al., 2007b). The introduction of PGRP protein into the haemolymph of *M. sexta* increases the production of AMPs but does not appear to have any effect on PPO activation, suggestive of a role in bacterial detection (Jiang, 2008). I show in chapter three that the knock down of PGRP results in a reduction of the up-regulation of two AMPs; attacin and moricin, and PPO (Figure 3.8 -3.10), proving that PGRP is essential for the up-regulation of these genes in response to infection. Furthermore, the knock down of PGRP has no effect on phagocytosis and formation of melanotic nodules (Eleftherianos et al., 2007b). All these results point to PGRP having a role in bacterial recognition and initiating the production of AMPs.

IML-2 has been shown to stimulate PPO activation and its depletion within plasma shown to inhibit clearance of *Serratia marcescens* and decreased survival of infection (Jiang, 2008). Knock down of IML-2 results in increased susceptibility to *P. luminescens* strain TT01, and this effect is stronger than either hemolin or PGRP (Eleftherianos et al., 2006a, , 2006b). This may be due to the protein's role in PPO activation but also it may have a function in initiating AMP production. The up-regulation of IML-2 is linked to the up-regulation of another immune-related gene: serine protease homologue 3 (SpH3). The knock down of SpH3 results in a reduction of up-regulation of AMPs in response to infection, but has no effect on the up-regulation of PRRs (I. Eleftherianos *et al* – unpublished data). This is suggestive of SpH3 being involved downstream of PRRs, perhaps involved in signalling.

Iron is an important nutrient for all living organisms with roles in DNA synthesis, photosynthesis and the activation of oxygen (Andrews et al., 2003). However, this is tempered by the fact that high levels are toxic to organisms through the formation of hydroxyl radicals. Hence many organisms have developed tightly regulated systems for the storage and use of iron within biological mechanisms. As such, pathogens find themselves in iron-restricted environments, and have had to develop mechanisms by which to source iron for their own needs. Many pathogenic bacteria produce siderophores; compounds that have a very high affinity for ferric iron. The

ferri-siderophores are then taken up by mechanisms on the outer membrane of Gram-negative bacteria in an energy-driven process provided by the TonB complex. Ferrous iron up-take is regulated by separate mechanisms that reside on the cytosolic membrane of Gram-negative bacteria.

Many animals, including insects use proteins for sequestering, transporting and storage of iron. *M. sexta* encodes two proteins for these processes; transferrin and ferritin. There have been reports to show that transferrin is up-regulated in response to infection (Andrews et al., 2003). This makes sense, as iron is such a valuable resource for pathogens, that insects would, as part of the immune response sequester iron so that it's not freely available to the pathogen. There is also evidence to show that ferritin has a role in the immune response of *Drosophila.* In chapter four, I show that both transferrin mRNA and protein levels are up-regulated in response to an immune challenge by *E. coli*. Up-regulation of ferritin mRNA and protein levels in response to the same challenge however does not occur. This indicates that transferrin may have some role within the immune response, but ferritin does not (Figure 4.1, Figure 4.2).

A range of iron up-take and storage knock-out mutants were donated to me by Robert Watson and David Clarke for the study of the role of transferrin within the *M. sexta* immune system. Of these mutants, those which the *exbD* or the *yfeABCD* gene knocked out were not pathogenic (Figure 4.3 – 4.14). The *exbD* gene encodes ExbD, part of the TonB complex (Watson et al., 2005). The *yfeABCD* gene encodes a ferrous iron up-take system with homology to a similar up-take system found in *Yersinia pestis*. This means that *P. luminescens* strain TT01 requires both ferric and ferrous iron as part of the infection process. This is similar to a mouse model of bubonic plague. A *Y. pestis* Yersinabactin (Ybt) mutant is unable to infect in the classical way via the lymph glands, however, if injected intravenously the mutant regains its pathogenicity. Ybt is a siderophore up-take mechanism meaning that *Y. pestis* requires ferric iron as part of the initial infection process. An Ybt^- yfe^- mutant loses all pathogenicity, indicating that ferrous iron is required for the second part of infection process (Perry et al., 2007).

Injection of ferric iron resulted in both knock-out mutants of *exbD* and *yfeABCD* regaining their pathogenicity. Injection of manganese on the other hand did not

reverse the loss of pathogenicity of the knock-out mutants (Figure 4.16 – 4.19). It is quite clear therefore that the acquisition of iron is preventing pathogenesis and not that *P. luminescens* strain TT01 requires *exbD* or *yfeABCD* for pathogenesis. It is interesting to note that although *P. luminescens* strain TT01 has another mechanism by which it can up-take ferrous iron; *feoAB*, that the presence of this does not compensate for the loss of *yfeABCD*. This could be due to the mechanisms being regulated differently, and that *P. luminescens* strain TT01 uses *feoAB* at a different point in its lifestyle (i.e. in pathogenesis with another insect or when contained within nematodes). Similarly, in the example above with *Y. pestis*, it too also contains a *feo* system that does not compensate for the loss of its *yfe* system (Perry et al., 2007).

Injection of dsRNA specific for transferrin or ferritin did not make the insect susceptible to *P. luminescens* strain TT01 knock-out mutants. It also did not affect the infection process of the wild-type strain either. Further investigation revealed that transferrin mRNA levels were unaffected by the dsRNA, while ferritin mRNA levels were reduced, the protein levels were unaffected. In this final experiment, both the original wild-type strain and the rifamycin (Rif) resistant parent strain of the knock-out mutants can be compared. Rif acts upon RNA polymerase and prevents RNA synthesis. It is quite apparent that this resistant strain has much less virulence than the wild-type, and this may reflect on the knock-out mutants' reduced ability to kill. As obviously RNA synthesis is involved in all parts of the cell cycle, it would be impossible to determine exactly what is lacking from the Rif-resistant mutant. It could be that this resistance amplifies the effect of the knock-out on the pathogenesis of *P. luminescens* strain TT01.

A feature of the work done for this thesis that needs to be discussed is the failure to obtain reproducible RNAi knock-down of gene expression in *Manduca sexta* caterpillars. Previous work in our laboratory was successful with this technique (Eleftherianos et al., 2006a; 2006b; 2007a; 2007b), and indeed I contributed to some of this work. The original knockdowns were well documented, but it has proved impossible to replicate these results, and since autumn 2007, the same RNAi techniques that were previously successful have given negative or at best equivocal results. The cause of this problem remains unknown despite much work attempting to trace it.

One hypothesis, which is difficult to test, is that the Bath colony of *Manduca sexta* may have become infected with a latent, symptomless virus or some other parasite that inhibits RNAi. Such an infection would be hard to detect (e.g. by PCR) unless the identity of the agent was known. Moreover, some viruses are known to possess genes that encode inhibitors of the RNAi machinery. A good example is the Flock House Virus (FHV), an α-nodavirus that is found in many *Drosophila* stock cultures, and which has recently been found to be present in a lepidopteran cell line as a latent and completely symptomless infection (Li et al., 2007). FHV encodes protein B2, a potent inhibitor of RNAi on its subgenomic RNA3 segment (Li et al., 2002). B2 has been shown to bind dsRNA, and thus prevent its binding to Dicer (Lingel et al., 2005). The replication of this single-stranded RNA virus requires a stage in which dsRNA is produced within the host cell, and therefore, the inhibition of the host's RNAi response to dsRNA is a requirement for the virus's success.

While study of the RNAi suppressing properties of FHV B2 has had beneficial results in terms of understanding, and possibly may even find application (Venter et al., 2008), if similar viruses occurred widely within animals, this might well explain why RNAi has characteristically been found to be an unpredictable phenomenon, that is hard to reproduce consistently in the lab outside of one or two tractable models like *C. elegans* (perhaps those that are not infected with such viruses). So far attempts to detect or eliminate any unknown virus have been unsuccessful (J. Garbutt, personal communication).

Fluorescent-activated cell sorting (FACS) is proving to be very useful for the study of insect immune cellular responses. It is a highly sensitive technique that allows for great resolution of interactions between cells (Tirouvanziam et al., 2004). In chapter 5, I developed a protocol for the use of FACS to investigate haemocyte behaviour in response to infection to both *E. coli* and *P. luminescens* strain TT01.

Phagocytosis is part of the cellular response against infection. *P. luminescens* strain TT01 excretes a variety of factors to prevent phagocytosis of itself along with other effectors to inhibit other cellular responses. This includes LopT, which is secreted

into a phagocyte by a Type III Secretion System (Goodrich-Blair and Clarke, 2007). This is a toxin that interferes with the actin cytoskeleton of the cell and will inhibit phagocytosis as a result. The cells reported to act as phagocytes are granular cells, although in *M. sexta* hyperphagocytes are reported to the majority of phagocytosis despite their small population. Peanut agglutinin (PNA) is a lectin reported to be specific for granular cells, and thus was chosen to be used to measure changes in haemocytes in response to infection. Unfortunately, there was not a clear distinction show in the fluorescence between granular cells and other cells. This was actually due to fact that PNA is able to bind to all activated haemocytes, and so would not be suitable for distinguishing between cell populations. A better solution would be the use of specific antibodies for the individual cell populations. MS13 and MS34 antibodies are specific for plasmatocytes whereas MAb 15D11 can be used to label granular cells (Nardi et al., 2003). These two cell types make the majority of the haemocyte population within *M. sexta* and thus being able to distinguish between them would be very useful.

Both granular cells and plasmatocytes are activated when a foreign surface is encountered (Lavine and Strand, 2002) so the experimental protocol which calls for bleeding into tubes and centrifugal washes might activate some of the cells, giving false readings. It might be a better idea to 'fix' the haemocytes using para-formaldehyde so that they are not activated when they come into contact with the foreign surfaces of the tube and pipette tips.

I was able to confirm that phagocytosis of *P.* luminescens strain TT01 by insect haemocytes does not occur by the use of both FACS and confocal microscopy. Furthermore, it was quite hard to find cells on samples that had been treated with *P. luminescens* strain TT01. This could be due to some loss of cells through washing as per the experimental protocol but as it was easier to find cells on the controls, this can be ruled out. Therefore this might due to some inhibitory factor secreted by *P. luminescens* strain TT01 that stops the proliferation of haemocytes. To further investigate the inhibitory effect of *P. luminescens* strain TT01, I pre-treated *M. sexta* with *P. luminescens* strain TT01 before injection with *E. coli*. This shows much less phagocytosis than the controls. But those that had been pre-treated with *E. coli* before injection with *E. coli* showed even less than this. This could not be due to inhibitory factors as this *E. coli* strain does not contain any. The lack of recovery of

colony forming units (CFU) of *E. coli* incubated in cell-free plasma taken from insects pre-treated with *E. coli* is almost certainly due to up-regulated humoral responses from the original pre-treatment and probably explains the lack of phagocytosis seen in Figure 5.12. It was a similar story for those bacteria incubated in cell-free plasma taken from those insects pre-treated with *P. luminescens* strain TT01 with very few CFU recovered (Figure 5.14). The story here is less clear though as *P. luminescens* strain TT01 is able to produce a broad spectrum antibiotic (Eleftherianos et al., 2007a). Also it is not known whether AMPs are up-regulated in response to *P. luminescens* strain TT01. It cannot be confirmed whether bacterial or insecticidal factors are responsible for the small recovery of CFU from this experiment.

The interactions between *M. sexta* and *P. luminescens* strain TT01 are many and varied. *P. luminescens* strain TT01 is able to inhibit many of *M. sexta* immune defences (Eleftherianos et al., 2007a) despite its recognition by PRRs, and thus ultimately render the defences futile. The pre-immunising of *M. sexta* by *E. coli* however allows the insect to arm itself with considerable defences that reverse the trend (Eleftherianos et al., 2006a). The basis of this defence lies in the up-regulation of PRRs of which PGRP plays a crucial role. It is essential for the up-regulation of important AMPs following immune challenge (Figures 3.8-3.10), which play a major role in the defence against *P. luminescens* strain TT01. The ability of pre-immunised cell-free plasma to kill most of the bacteria within one hour of exposure (Figure 5.14) suggests a high activity or concentration of humoral responses, that does not allow the cellular response to initiate (Figure 5.12). The role of transferrin in this response still has to be elucidated though. The protein seems to be up-regulated from about 18 hours after infection (Figure 4.2), which is the time between immune challenges of *E. coli* and *P. luminescens* strain TT01 in our experimental protocol (Eleftherianos et al., 2007b). Given that iron acquisition plays a vital role in the pathogenesis of *P. luminescens* strain TT01 (Figure 4.16, Figure 4.18), could the up-regulation of transferrin and the proposed sequestering of iron away from the bacteria have a role in the pre-immunised insect increased ability to fight *P. luminescens* strain TT01 infection.

Further work

It would be interesting to see if the knock down of PGRP had an effect on the up-regulation of other AMPs. Also, the knock down of one or two other PRRs would perhaps reveal their role within humoral responses It appears at the moment that hemolin mediates the cellular response (Eleftherianos et al., 2007b) while PGRP is primarily responsible for the activation of AMPs. The role of IML-2 appears to lie in PPO activation (Kanost et al., 2004), although there is a suggestion that it might also act in AMP up-regulation. Knock down of any or a combination could further elucidate their roles according to AMP production. Also to investigate whether there is a specific response to Gram-negative or Gram-positive bacteria, qPCR could be used to study the strength of the response to different stimuli. This could also be achieved a little more crudely by pre-immunisation experiments, i.e. injecting harmless Gram-positive bacteria, and seeing if that protects against *P. luminescens* strain TT01 and vice versa.

It would also be interesting to repeat the iron knock-out mutant experiments with a parent strain of *P. luminescens* strain TT01 that is not so hampered in its virulence. This might further prove the importance of iron to pathogenesis. Also, as the *exbD* gene knock-out probably has effects on many mechanisms for the up-take of iron (Watson et al., 2005), it would be good to narrow it down to which one or two mechanisms is responsible for the up-take of ferric iron in the pathogenic stage of *P. luminescens* strain TT01 lifecycle.

The role of FACS in studying immunity could be a strong one (Tirouvanziam et al., 2004). A protocol needs to be developed to accurately identify the various haemocytes. The use of specific antibodies would help greatly here. It is probable that haemocytes are the first to encounter bacteria and it is probable that PGRP and hemolin proteins expressed by these haemocytes are responsible for the initiation of the immune response. FACS might be able to identify the signalling factors responsible and which cells express them.

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Appendix 1 – Recipes

LB
1L of Filtered Water
10g of Sodium Chloride
10g of Tryptone
5g of Yeast Extract

LB with Agar
1L of Filtered Water
10g of Sodium Chloride
10g of Tryptone
5g of Yeast Extract
15g ofAgar

TAE Buffer (50x)
242g of Tris-Base
57.1mL of Glacial Acetic Acid
100 mL 0.5 M EDTA
Water up to 1000mL

Anticoagulant Saline for *Manduca sexta*
6.6g of Sucrose
10 mL of 10x Salt Solution (2.34g of Sodium Chloride, 32.08g of Potassium Chloride, 73.2mL of Magnesium Chloride Solution (50%w/v) Water up to 1L)
50mL Distilled Water, mix until Sucrose dissolved
1mL of 100x Buffer Solution (Make 500mL 150mM Na_2HPO_4 (11.70g) and 150mM NaH_2PO_4 (10.65g) Mix solutions to make final pH of 6.9)
Make up to 100mL with Distilled Water
Adjust pH to 4.5 with HCl or KOH

PBS
Dissolve 1 tablet per 100mL Distilled Water

TPBS
Dissolve 1 tablet per 100mL Distilled Water
Add 500µL/L Tween20

Sample Buffer
15.1g/L Tris
46g/L Sodium Dodecyl Sulphate
40mL/L Glycerol
0.01% Bromophenol Blue
5% w/v β-mercaptoethanol

Towbin Buffer
3g/L Tris
14.1g/L Glycine
200mL/L Methanol

Make up to 1000mL with Distilled Water

Running Buffer (10x)
30.3g/L Tris
144.0g/L Glycine
10.0g/L Sodium Dodecyl Sulphate
Make up to 1000mL with Distilled Water

Tris pH8.8 (200mL)
36.33g Tris (1.5M)
1.6g of Sodium Dodecyl Sulphate
200mL of Distilled Water
Adjust pH to 8.8 with Concentrated HCl

Tris pH6.8 (200mL)
12.12g of Tris (0.5M)
0.8g of Sodium Dodecyl Sulphate
Make up to 200mL with Distilled Water
Adjust pH to 6.8 with Concentrated HCl

SDS-PAGE 12% Separating Gel
4.0mL Acrylamide
3.3mL Sterile Distilled Water
2.5mL Tris pH8.8
100µL 10% Sodium Dodecyl Sulphate
100µL 10% APS (0.1g in 1mL Sterile Distilled Water)
4µL TEMED

SDS-PAGE 5%Stacking Gel
670µL Acrylamide
2.7mL Sterile Distilled Water
500µL Tris pH6.8
40µL 10% Sodium Dodecyl Sulphate
40µL 10% APS (0.1g in 1mL Sterile Distilled Water)
4µL TEMED

Appendix 2 – Primer Sequences and Product Size

PGRP-1A RT-PCR (Product size: 516bp)
Forward 5'to 3': ACGGTATCACTTCCGTCCAC
Reverse 5' to 3': CATTCTGGCATCTCCTGAT

PGRP-1A qPCR (Product Size: 124bp)
Forward 5'to 3': TGAAGTGTGGCGTTGACAAT
Reverse 5' to 3': CATTCTGGCCATCTCCTGAT

rpS3 (Product Size: 186bp)
Forward 5' to 3': CTGGCTGAGGATGGCTACTC
Reverse 5' to 3': TTTCTCAGCGTACAGCTCCA

Attacin B (Product Size: 341bp)
Forward 5' to 3': GGTCACGGAGCTACTCTTAC
Reverse 5' to 3': TTGGGCATCTCGAACTTCTT

Moricin 2 (Product Size: 327bp)
Forward 5' to 3': TCGCGTGAGGATGGCTACTC
Reverse 5' to 3': CAGAAGATTCCGAAGGGAGA

ProPhenoloxidase (Product Size: 886bp)
Forward 5' to 3': AAACAACTCCCAAACGATGC
Reverse 5' to 3': TGTGCATGTTGTTGTGGATG

Transferrin (Product Size: 840bp)
Forward 5' to 3': TCTAAGTGCCGGGCTATGTC
Reverse 5' to 3': TGGATGGTCTTGAACTTCTCG

Ferritin (Product Size: 614bp)
Forward 5' to 3': CCGACACTTGCTACCAGGAC
Reverse 5' to 3': ACTCGTCGAAAACGTACAGG